SINGULAR AND COMBINED EFFECT OF POSTHARVEST TREATMENTS ON VIABILITY AND REPRODUCTIVE ABILITY OF *PHYLLOSTICTA CITRICARPA* INFECTIONS

by

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SUMMARY

Citrus is one of the most important fruit crops globally and is currently being produced in over 100 countries. South Africa is one of the biggest shipping exporters of fresh citrus, with approximately 40% exported to European markets. Packhouses have rigorous export quality control programmes to maintain quality and prevent postharvest decay during the shipping period.

Citrus black spot (CBS) (caused *by Phyllosticta citricarpa* (McAlpine) van der Aa) is mostly a cosmetic disease that reduces the aesthetic quality of fruit and does not cause postharvest decay. However, *P. citricarpa* is regarded as a quarantine organism in certain countries, and despite scientific evidence to the contrary, trade restrictions are imposed, such as the zero tolerance for CBS lesions on fruit exported to European Union. Whilst fruit may be exported from areas where CBS occurs, very strict preharvest control programmes must be followed to ensure fruit production in orchards meet the zero tolerance requirements. The biggest danger surrounding CBS is the presence of latent, asymptomatic infections in harvested and packed fruit, which can sometimes manifest on the fruit long after packhouse treatment, cold storage and shipping.

Previous studies have indicated that postharvest treatments delay symptom expression and control CBS by reducing lesion and pycnidiospore viability. The objective of this study was to evaluate the effect of more recent protocols and fungicides used in packhouses, as well as alternative fungicides, against latent CBS infections, including the reproductive potential of the lesions.

Fruit with CBS lesions, as well as asymptomatic fruit with latent infections, were subjected to standard packhouse sanitation, fungicide treatment and cold storage (singularly and combined), and incubated at conditions that enable expression of latent infections. The full packhouse treatment along with storage period gave significantly control of latent infections. The over all reproductive ability of lesions were very low, with less than 2.1% of all lesion that formed on both Valencia's and Eureka lemons developing pycnidia. Three alternative single treatments showed potential to control latent infections: FLU, potassium sorbate and Propirly 270 EC (PPZ + PYR). Treatment with (respectively) FLU and Propirly 270 EC resulted in moderate to significant control of latent infections on both Valencia oranges and Eureka lemons. Potassium sorbate moderately controlled latent CBS infections in both Valencia oranges and Eureka lemon trials. The combined epidemiological requirements for pycnidiospore release along with results from trials conducted in the current study indicate that harvested fruit is not an epidemiologically significant pathway for the spread of CBS.

OPSOMMING

Sitrus is wêreldwyd een van die belangrikste vrugtegewasse en word tans geproduseer in meer as 100 lande. Suid-Afrika is een van die grootste uitvoerders van vars sitrus, met ongeveer 40% van die produksie uitgevoer na Europese markte. Pakhuise is onderworpe aan streng programme om uitvoergehalte te beheer deur die handhawing van hoë vrugkwaliteit en voorkoming van na-oes vrugbederf.

Sitrus swartvlek (SSV) (veroorsaak deur *Phyllosticta citricarpa* (McAlpine) van der Aa) is hoofsaaklik 'n kosmetiese siekte wat die uitvoerkwaliteit van vrugte verswak. Alhoewel dit nie na-oes bederf veroorsaak nie, word dit egter in sommige lande beskou as ‘n kwarantyn organisme. Ten spyte van wetenskaplike bewyse tot die teendeel, word handelsbeperkinge teen SSV vrugte steeds opgelê, byvoorbeeld die nul toleransie van SSV letsels op vrugte uitgevoer na die Europese Unie. Produksie areas waar SSV voorkom volg streng vooroes programme om te verseker dat vrugte uit sulke boorde aan hierdie nul toleransie vereistes voldoen. Die grootste gevaar rondom swartvlek is die teenwoordigheid van latente, asimptomatiese infeksies op geoesde en verpakte vrugte Sulke infeksies word soms eers uitgedruk lank na na-oes behandeling, verkoeling en verskeping van die vrugte.

Vorige studies het aangedui dat na-oes behandelings simptoomuitdrukking vertraag, met gevolglike swartvlekbeheer deur die vermindering van letseluitdrukking en die lewensvatbaarheid van piknidiospore. Die doel van hierdie studie is om die meer onlangse pakhuis protokolle en swamdoders, asook alternatiewe swamdoders, te evalueer vir hul uitwerking teen latente swartvlek infeksies en hul effek op die reproduktiewe potensiaal van letsels.

Vrugte met swartvlekletsels, asook asimptomatiese vrugte met latente infeksies, was onderwerp aan standaard pakhuissanitasie, swamdoderbehandelinge en koelkameropberging (alleenstaande en gekombineer). Daarna was dit geïnkubeer onder omstandighede wat simptoomuitdrukking bevoordeel. Die volledige pakhuisbehandeling, tesame met ‘n kouestoor tydperk, het aansienlike beheer van latente infeksies gegee. Oor die algemeen was die voortplantingsvermoë van letsels baie swak, met minder as 2.1% van alle letsels op beide Valencia en Eureka suurlemoene wat uiteindelik piknidia ontwikkel het. Drie alternatiewe alleenstaande behandelings het potensiaal getoon vir die beheer van latente infeksies: FLU, kaliumsorbaat and Propirly 270 EC (PPZ + PYR). FLU en Propirly 270 EC het matige tot aansienlike beheer oor latente infeksies op beide Valencia lemoene en Eureka suurlemoene uitgeoefen. Kaliumsorbaat het matige beheer oor latente swartvlek infeksies op beide Valencia

lemoene en Eureka suurlemoen uitgeoefen. Die kombinasie van epidemiologiese vereistes vir piknidiospoor vrystelling, tesame met die resultate van hierdie proewe, dui daarop dat ge-oesde vrugte is nie 'n noemenswaardige epidemiologiese weg bied vir die verspreiding van swartvlek nie.

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CONTENTS

CHAPTER 1

A review of the epidemiology of citrus black spot, with specific reference to options for postharvest chemicals that can be used to control latent infections

INTRODUCTION

Citrus (*Citrus sinensis* (L.) Osbeck) is one of the most important fruit crops globally (CGA, 2015). It is produced in over 100 countries, in areas mainly located within the 40° northern and southern latitudes (Spiegel-Roy and Goldschmidt, 1996), and was first established in South Africa in 1652, shortly after the arrival of Jan van Riebeeck (Obagwu and Korsten, 2003). The citrus export market in South Africa was officially established in 1907 (Stanbury, 1996), and has since been expanding each year (CGA, 2016). South Africa is a leading global exporter of fresh citrus since 2006, and for the last 15 years the second biggest exporter of fresh citrus fruit in the world with close to 1.7 million tons exported in 2015 (CGA, 2016). Only 8% of the income generated by citrus production in South Africa comes from citrus sold on local markets and from processing, while 92% of citrus earnings comes from the export sector (CGA, 2016). In 2015 South Africa produced 118 million cartons (15 kg equivalents) of fresh citrus fruit, of which 40% was exported to European markets (CGA, 2016). In terms of volumes taken, the most important export markets for South Africa are Europe, United States of America, Asia, Middle East, Far East, Canada, Russia and United Kingdom, but this is variable depending on citrus type (CGA, 2016).

Although being a major exporter, we are only the eleventh largest producer of citrus after China, Brazil, India, USA, Mexico, Spain, Egypt, Turkey, Italy and Iran (Key Industry Statistics for Citrus Growers, 2016). Within each of these countries different citrus types are produced, mainly grapefruit, lime, lemon, sweet orange and mandarin (Davies and Albrigo, 1994; CGA, 2016). With the evolution of citrus production over the years, various new cultivars have been developed within each citrus type, which differ in quality, size, shape, growing conditions and season of maturity (Nicolosi, 2007). Cultivars are selected to best adapt to the specific environmental conditions and soil factors in a specific area.

Due to the economic contribution, the citrus export trade is very important to South Africa's economy, generating R 13.2 billion in 2015/16 (CGA, 2016). The citrus industry is furthermore responsible for employing 14% of the agricultural job market, employing approximately 125,000 workers (CGA, 2016). Maintaining these different export markets requires adherence to the specific standards imposed by each market (European Commission, 1998). All the factors influencing quality and phytosanitary status of the fruit need to be managed properly, else trading routes can be lost, leading to a decrease in the income generated through export (Baayen *et al*., 2002). Citrus black spot, caused by *Phyllosticta citricarpa* (McAlpine) van der

Aa, is one of the factors endangering our citrus export to the European markets. The disease is mainly cosmetic, but heavy infections can cause early fruit drop in countries with extremely high disease pressure in the orchards (Brazil) (Truter, 2010). In South Africa, CBS rarely causes crop loss, but the presence of the disease in production regions (Carstens *et al*., 2012) or the presence of CBS lesions on fruit disqualify access to certain markets, in particular the European Union (EU) that has a zero tolerance for CBS-infected fruit.

The EU's zero tolerance for CBS has been challenged by South Africa (South African CBS PRA, 2000-2009), and the EU's recent pest risk assessment for *P. citricarpa* (EFSA, 2014) was criticised by an international panel of CBS experts (CBS Expert Panel, 2013; 2014; 2015). The CBS Expert Panel (2013, 2014, 2015) agreed with earlier pest risk assessments, conducted by South Africa and USA (USDA-APHIS, 2010), in which it was concluded that fruit is not a realistic pathway for CBS to enter, establish, spread or have significant economic impact within the EU.

ECONOMIC IMPACT AND SHORT HISTORY OF CBS

Apart from market access concerns, the economic loss due to CBS infection is mainly attributed to cosmetic rind defects, which reduces the quality for the fresh fruit market (Kotzé, 1981). *Phyllosticta citricarpa* can remain a latent infection that might be expressed as CBS lesions much later. This was shown by isolation of the fungus from healthy fruit tissue (Azevedo *et al*., 2000; Araújo *et al*., 2001; Glienke-Blanko *et al*., 2002). Fruit that are heavily infected with *P. citricarpa* are used for juice production, which yields a much lower income per ton produced (Cobb, 1897; Calavan, 1960; Kellerman and Kotzé, 1977). As early as 1895, A.H. Benson noted that CBS infection in fruit led to significant losses in citrus production areas in Australia (Truter, 2010). This happened again in 1939 when there was a severe CBS epidemic in New South Wales. In 1945 the citrus production areas in the northern provinces of South Africa were badly infested with CBS, resulting in 90% of fruit produced in unsprayed orchards to be deemed unfit for export (Sutton and Waterson, 1966). As early as the 1960's worldwide economic losses due to CBS was speculated to have amounted to millions of dollars (Calavan, 1960). One of the most severe problems that contribute to preharvest losses is premature fruit drop and waste in severely infected orchards (Kiely, 1970).

Postharvest CBS losses can be attributed to latent or asymptomatic infections that can develop CBS symptoms during export (Kiely, 1948a; Loest, 1958; Smith, 1962; Brodrick, 1969). Additionally, treatment programs to control CBS in the orchards are extremely costly (Cobb, 1897; Kotzé, 1961), but if the orchards are left untreated the entire crop could be lost due to CBS (Seberry *et al*., 1967). In most citrus production areas where CBS is prevalent, production will be impossible without an effective CBS control program (Smith, 1996). Trials

conducted by Schutte *et al*., (2003) showed that two applications comprising of azoxystrobin and mancozeb, during mid-November and mid-January, reduced CBS incidence by 95 – 100%. Results from earlier studies conducted by Kellerman and Kotzé (1977) also showed high efficacy of preharvest fungicide applications.

Whilst very high levels of preharvest control of CBS can be achieved, the current zero tolerance phytosanitary restrictions enforced by the European Union makes it increasingly difficult to comply, especially for countries with long trading routes (Baayen *et al*., 2002). With all the expenses incurred to meet export requirements, refusal of an infected shipment by an importing country is a severe economic setback to the exporter, normally entailing rerouting or return of the affected shipment or worse, the fruit being destroyed. Furthermore, non-compliance to phytosanitary regulations might compromise complete export programs, with market access being denied due to the perceived risks of continued trade (CGA, 2016).

EPIDEMIOLOGY OF *PHYLLOSTICTA CITRICARPA*

Phyllosticta citricarpa is a eukaryotic fungus that is classified under the phylum Ascomycota and the family Botryosphaeriaceae. In 1899, McAlphine was the first to describe the anamorph of the black spot fungus as *Phoma citricarpa*, however Cobb already described the endophytic and latent nature of *P. citricarpa* in 1897. In 1948 the teleomorph was described by Kiely and reclassified as *Guignardia citricarpa*. The anamorph was then later reclassified by Van der Aa in 1973 as *P. citricarpa* (McAlpine). According to the modern, accepted nomenclature system, however, both anamorph and teleomorph carries the same name, therefore the name was changed to *P. citricarpa* (Wikee *et al*., 2011). Even before the name change, it was considered that there were two distinct strains of *Guignardia citricarpa*: one that is pathogenic to citrus and the other non-pathogenic with a wide host range (Kiely, 1948a; Kotzé, 1981). This pathogenicity of *P. citricarpa* was proven, while *G. mangiferae* A.J. Roy was determined to be non-pathogenic, only causing latent, non-symptomatic infection on citrus fruit (Meyer *et al*., 2001; Baayen *et al*., 2002; Bonants *et al*., 2003). Baayen *et al*. (2002) used several morphological characteristics in his assessments (growth, colony type, and production of pycnidia in ascocarps in culture), as well as sequences of the ITS region and amplified fragment length polymorphisms to distinguish between the two species. The widespread non-pathogenic strain on citrus has finally been designated as *Phyllosticta capitalensis*, a taxon distinct from *G. mangiferae* (Glienke-Blanco *et al.* 2011; Wikee *et al*., 2013).

Almost all commercial citrus species are susceptible to CBS, but lemons and Valencia oranges are the most susceptible. Citrus black spot has also been found on citron, pomelos and mandarins, with significant losses reported on grapefruit and lemons (Kiely, 1948a;

Brodrick, 1969; Kiely, 1970). Lemons are particularly sensitive to CBS, and therefore the first observation of the disease in a previously unaffected area is often made on this citrus type (Kiely, 1948b; Kotzé, 2000). Persian limes, and sour orange and its hybrids are thought not to be susceptible (Kotzé, 1981), while rough lemons are thought to be tolerant (Wager, 1952).

Life cycle

Phyllosticta citricarpa has two distinct life stages, sexual and asexual, each producing unique spores that can spread the disease (Kotzé, 1981; Kiely, 1948a). Each one of these stages are discussed below.

Sexual stage

The sexual stage of *P. citricarpa* disease cycle initiates when infected leaves drop and accumulate on the orchard floor during the late winter to early spring. Although the leaves are infected while still on the tree, the infection remains latent until after leaf abscission (Kiely, 1948b; Whiteside, 1965). Ascospores are exclusively produced on the infected leaf debris (Sutton and Waterson, 1966), and have never been observed on fruit, twigs or leaves still attached to the tree (Kiely, 1948b; Kotzé, 1963, 1981; Van der Aa, 1973). Ascospores are the primary inoculum for CBS, and are produced in groups of eight inside sack-like structures called asci, carried in pseudothecia (Kotzé, 1963; McOnie, 1965; Truter *et al*., 2007). The pseudothecia are produced in groups of two (200-240 µm) or three (340-360 µm) (Kiely, 1948b) and are formed sub-epidermal. Although it has no stroma or distinct beak, an ostiole is present at maturity (Kiely, 1948b; Van der Aa, 1973).

The maturation of fruiting structures and release of ascospores is greatly influenced by temperature (mild to warm) and alternating wetting periods (rain, irrigation water or heavy dew) (Kiely, 1948b; Kotze,1963; McOnie, 1964b, 1965; Lee and Huang, 1973; Fourie *et al*., 2009; Truter, 2010). Fully formed pseudothecia structures are visible on the ventral and dorsal surfaces of the leaf debris approximately 30 to 180 days after leaf drop. Kiely (1948a) showed that dead leaves produce ascospores for several months, even when leaves were in an advanced stage of decomposition. Released ascospores are distributed over short distances by wind until they are deposited on susceptible leaves or fruit (Kotzé, 1963, 2000; Garrán, 1996). These ascospores are distributed by wind, and will germinate on the surface of a fruit/leaf within a 15 hour wetting period at an optimal temperature of 27°C (Kiely, 1948b; Kotzé, 1963; McOnie, 1964b, 1967). Germination and infection of ascospores require the presence of free surface water (Kiely, 1948b).

Upon penetration of the cuticle, the germination tube will expand into a small mass of mycelium to form a latent infection (McOnie, 1965). The critical period for fruit infection lasts from fruit set to 4-5 months later (Kiely, 1948b; Kotzé, 1963; McOnie, 1964b; Schutte *et al.*, 1996), while leaves are susceptible up to 10 months after formation (Truter *et al*., 2007). The infections can remain latent for 6 months or longer, and black spot symptoms develop on fruit usually after colour break when fruit matures (Kiely, 1948b; Kotzé, 1963, 1981).

Asexual stage

Pycnidiospores are asexual spores produced in pycnidia, which are small, melanised, globular structures, usually encased in host tissue. There are various CBS lesion types present on citrus fruit, of which only hard spot (Darnell-Smith, 1918; Kiely, 1948b; Wager, 1952; Kotzé, 2000), freckle spot (Kotzé, 2000) and virulent spot develop pycnidia (Kiely, 1948b; Kotzé, 2000). Although more lesion types have been identified, only five types have been confirmed as typical in the South African citrus industry by Truter (2010). Pycnidia will develop within the hard spot, freckle spot and virulent spot lesions on infected fruit, leaves or twigs (Kiely, 1948b; Wager, 1952; McOnie, 1964b; Garrán, 1996; Kotzé, 2000).

The pycnidiospores develop through mitotic division and are released through the ostiole, in a gelatinous mass that rely on running water for distribution when mature (McOnie, 1964b; Kotzé, 1981; Whiteside, 1993; Garrán, 1996). These oozed pycnidiospores can only be distributed over short distances (<1 m) by water, from fruit in the top of the tree canopy to lower hanging fruit and leaves (Kiely, 1948b; McOnie, 1965; Spósito *et al*., 2008). According to Kotzé (1963) the germination of pycnidiospores follows during a wetting period of at least 12 hours, at optimal temperatures of 25°C. In favourable environmental conditions, pycnidia production can be continuous inside a lesion (Kiely, 1948b).

Other sources of inoculum

Kiely (1949) reported that latent mycelia present in infected citrus trees can also act as a source of inoculum. Calavan (1960) stated that infected propagation material moving from infected to new uninfected citrus production areas, could successfully introduce *P. citricarpa* into such previously uninfected orchards. In the past, before optimal control measures were put in place, CBS was spread to new unaffected areas by means of infected nursery material (Kiely, 1949; Wager, 1952).

It was feared that various non-citrus species indigenous to Australia and South Africa may host *P. citricarpa* and act as a natural source of inoculum (Kiely, 1948a, 1948b; Wager, 1952). However, McOnie (1964b) found that all the *Guignardia* isolates collected from non-citrus

hosts were identified to be *G. mangiferae*. This was later confirmed through work done by Meyer *et al*. (2001), and Baayen *et al*. (2002), using a variety of molecular identification techniques.

Distribution of CBS in South Africa

The first record of *Phyllosticta citricarpa* in South Africa dates back to 1929, in the area surrounding Pietermaritzburg (Doidge, 1929). Since then it has been found in several other summer rainfall areas of South Africa. In a comprehensive study done over a period of 15 years, the Western Cape, Northern Cape and the Free State provinces were all identified as CBS free areas (Carstens *et al*., 2012). Even though propagation material contaminated with *Phyllosticta citricarpa* have been used in these areas, they have remained free of CBS due to the specific environmental requirements of the organism for infection (European Commision, 1998; Mabiletsa, 2003). To infect, the organism needs a high temperature (Kotzé, 1963; McOnie, 1967; Brodrick and Rabie, 1970) and a long wetting period, which is only found in summer rainfall areas. The organism will therefore not be able to establish in areas with cooler, winter rainfall or Mediterranean-type environmental conditions (Kotzé, 1963; McOnie, 1964c; Smith, 1996) as was also demonstrated using epidemiological models (Kiely, 1948b; Kotzé, 1981; Paul *et al.*, 2005; Fourie *et al*., 2013; Yonow *et al*., 2013; Magarey *et al*., 2015).

CBS disease has also been recorded in several other areas, in Asia (Fujian, Guangdog, Guangxi, Hong Kong, Jiangsu, Sichuan, Yunnan, Zhejiang, Indonesia, Java, Philippines, Taiwan) (CABI/EPPO, 2012; EPPO, 2014), Africa (Kenya, Nigeria, Mozambique, Swaziland, Uganda, Zambia, Zimbabwe) (Whiteside, 1965; CABI/EPPO, 2012; EPPO, 2014), Central America and Caribbean (Cuba) (CABI/EPPO, 2012; EPPO, 2014), South America (Amazonas, Brazil, Espirito Santos, Minas Gerias, Rio de Janeiro, Parana, Santa Catarina, Sao Paulo) (CABI/EPPO, 2012; EPPO, 2014), Europe (Russian Far East) (CMI, 1990) and Oceania (Australia, New South Wales, Queensland, Victoria) (CABI/EPPO, 2012; EPPO, 2014).

Symptoms and lesion types

CBS is characterized by the lesions that develop on maturing fruit of sweet orange, lemon, grapefruit and other citrus varieties (McOnie, 1964a). Infections on leaves and stems mostly remain latent and symptoms are rarely discernible on the tree, but pycnidia developing in these lesions can serve as a secondary source of inoculum (Kellerman and Kotzé, 1977; Whiteside, 1993). Older lesions on leaves have symptoms that include small, round, necrotic, sunken spots with a grey centre that is surrounded by a dark-brown ring (Kiely, 1949; Kotzé, 1963). Younger leaf lesions are generally small, reddish and slightly raised - sometimes with a yellow

halo (Truter, 2010). Foliar and stem lesions are more commonly found on lemons than other cultivars (Whiteside, 1993; Truter, 2010). Fruit lesions are much easier to identify, and a variety of symptoms has been observed: (1) red spot; (2) hard spot; (3) false melanose/speckled blotch; (4) virulent spot; or (5) cracked spot. However, these symptoms can be very difficult to categorise in a specific class because of the variability (Calavan, 1960; McOnie, 1965; Kotzé, 1963, 1981, 2000; Whiteside, 1993; Garrán, 1996).

Red spot

Truter (2010) was the first to formally describe red spot lesions, but they have been mentioned in previous publications (Kotzé, 1963; McOnie, 1967; Korf, 1998; Bonants *et al*., 2003). Red spot is usually the first postharvest symptom to develop on packed fruit. This lesion requires a higher temperature and a longer incubation period to develop (Truter, 2010). Lesions are reddish, round, sunken depressions that form on the fruit rind (Fig. 1), and the pathogen can be isolated from them with ease (Truter, 2010). Pycnidia can form in red spots, and as the lesions become older they develop into hard spots (McOnie, 1967).

Hard spots

The presence of hard spots (Fig. 2) is the most prevalent and diagnostic symptom of CBS in all regions where black spot occurs. Development of hard spots initiates as the fruit becomes more mature, and normally appears when the fruit starts to colour up (Garrán, 1996; Whiteside 1993). The lesions can differ in size (3-10 mm) but normally appear as a circular depressions. These lesions can appear brick red with a tan to grey centre, have a distinct brown to black margin (Kotzé, 1963; McOnie, 1967), and contain pycnidia (Whiteside, 1993; Korf, 1998). Identification of the pycnidia requires skill and caution, as it can easily be confused with the acervuli of *Colletotrichum gloeosporioides* (Kotzé, 1963; Schutte, personal comm.). Hard spot lesions tend to appear more frequently on the side of the fruit exposed to the sun during fruit development (Whiteside, 1993).

False melanose or speckled blotch

Speckled blotch, better known as false melanose (Fig. 3) appear as several small, black to brown raised lesions that covers most of the fruit surface (Kiely, 1948b). These lesions do not contain any pycnidia or any other reproductive structures (Garrán, 1996; Korf, 1998). Individual false melanose lesions are mostly observed on green fruit, and can coalesce as the season progress. Closer to the end of the season false melanose symptoms can evolve into hard spot type symptoms (Kotzé, 1963).

Virulent spot

Virulent spots can develop from a coalescence of freckle spot lesions (Kiely, 1948a), and are mostly found on heavily infected, mature fruit toward the end of the season. The lesions normally appear sunken into the flavedo, with an irregular shape (Fig. 4). On asymptomatic fruit, virulent spot starts as small, somewhat sunken spots with irregular form and recessed centres (Calavan, 1960). These lesions initially have a distinct reddish colour, but as they mature can turn brown to black with a leathery texture, and can eventually cover the entire fruit surface (Kiely 1948a; Kotzé, 1963; McOnie, 1965). Expanded, mature lesions normally develop much later in the season on mature fruit, with the dark colour in part due to black pycnidia in the centre of lesions (Kotzé, 1981). The necrotic, suberized plant tissue further accounts for the dark colour of the lesion.

Cracked spot

These lesions develop on very old fruit (older than six months, physiologically compromised fruit) and are characterized by variable sizes of superficial lesions that appear to be cracked. The spots can form individually or in groups, and do not contain any pycnidia (Kiely, 1948b; De Goes *et al*., 2000, Truter, 2010) (Fig. 5)

PRE-HARVEST CONTROL OF CBS

The control of citrus black spot primarily needs to happen in the orchard. Achieving successful control requires integrated control to be applied, incorporating both chemical and cultural control practices.

Cultural control

Cultural control focuses on sanitation of the orchard along with managing general tree health. Removal of leaf litter from the orchard floor reduces the ascospore inoculum greatly, and if implemented as a long-term strategy it can be as effective as fungicide sprays (Truter, 2010). It is also important, especially for lemons, which has more than one fruit set per season, to remove late-hanging fruit to reduce pycnidiospores (Calavan, 1960; Kiely, 1969, 1970; Kotzé 1996). In an orchard infected with CBS, lesion manifestation usually first occurs on fruit from trees with low vigour (Kotzé, 1981). This emphasises the importance of maintaining tree vigour to minimise tree susceptibility to CBS infection (Calavan, 1960; Kiely, 1971; Loest, 1968).

Chemical control

The critical period for application of fungicides to control CBS on fruit is during the period of fruit susceptibility, which is the first 4-5 months after initial fruit set (Kiely, 1948b; Kotzé, 1963; Garrán, 1996; Schutte *et al*., 1997; Schutte, 2002; Schutte *et al.*, 2003; Miles *et al.*, 2004). These chemical applications have a preventative purpose, protecting the fruit against initial infection (Schutte *et al.*, 1997). Unfortunately, even timeously applied fungicides can still give variable results due to climatic conditions, spray coverage and susceptibility of cultivar (Kiely, 1969, 1970, 1971; Calavan, 1960). It is therefore always advisable to use chemicals in combination with other control practices.

The first fungicide used in preharvest control of CBS was the Bordeaux mixture (Benson, 1895; Cobb, 1897; Kiely, 1948b, 1950), but it was later discovered that regular application of this mixture led to copper toxicity, and alternatives had to be found (Kotzé, 1964). Following that, zineb (zinc ethylene bisdithiocarbamate, belonging to the dithiocarbamate group) and mancozeb (manganese ethylene bisdithio-carbamate) was successfully applied (Kotzé, 1964). These new fungicides were as effective as the copper fungicides, but had better activity than copper formulations, that resulted in rind stippling, retarded fruit colouring, and caused rind defects after frequent use (Schutte *et al*., 1997; Kellerman, 1976; Kellerman and Kotzé, 1977). Efficacy of the applied fungicides was further enhanced with oil additives to improve coverage of the fruit (McOnie and Smith, 1964; Kellerman, 1976; Kellerman and Kotzé, 1977).

Carbamates were eventually complimented with benomyl [methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate] that had both a preventative and curative action (Kiely, 1971; Kellerman and Kotzé, 1977). However, due to exclusive use of benomyl in orchards *P.citricarpa* developed resistance against the chemical by the early 1980's (Herbert and Grech, 1985; De Wet, 1987). This resistance resulted in the need for a new fungicide to replace it, and strobilurins showed a lot of promise (Schutte *et al*., 1996; Tollig *et al*., 1996; Schutte *et al*, 2003). Trials with strobilurins indicated that protective, curative and eradicative activities against CBS were possible. Furthermore, strobilurins residues appeared to have longer lasting control (Gold and Leinhos, 1995). To prevent the development of resistance against agricultural fungicides, it is important to protect their action through combination and rotation, for instance alternating mancozeb and strobilurins with a copper compound (Kellerman and Kotzé, 1977; Miles *et al*., 2004; Schutte *et al*., 2003).

POSTHARVEST FUNGICIDES CONSIDERED FOR CONTROL OF LATENT CBS INFECTIONS

Standard postharvest fungicides and chemicals

Imazalil

With blue and green mould resistance against benomyl and thiabendazole (TBZ) by the 1970's, a new fungicide was needed to replace them (McCornack and Brown, 1977). Imazalil (IMZ) was tested for blue and green mould control, and was found to be very effective in controlling TBZ and benomyl resistant strains (Harding, 1976; Bus *et al*., 1991; Erasmus *et al.,* 2013). IMZ was also found to have some controlling effect on other pathogens, specifically alternaria rot and stem-end-rot (Laville *et al*., 1977; McCornack and Brown, 1977).

Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy-ethyl]-1*H*-imidazole) is a FRAC code 3 fungicide, and acts as a sterol demethylation inhibitor of ergosterol biosynthesis through inhibition of the P450 enzyme, lanosterol 14α-demethylase (Siegel and Ragsdale, 1978; Hamamoto *et al*., 2000; FRAC, 2016). This group of fungicides do not have an inhibiting effect on the initial development of fungi, including spore germination, initial cell growth and the colony dry weight increase, but rather causes irregularities in the growth patterns of fungal colonies by changing the cells' morphology through inhibition of ergosterol biosynthesis (Siegel, 1981). Ergosterol is only found in the fungal cell membrane, which makes IMZ a selective fungicide that only affects fungi and not any higher eukaryotes such as plants and insects (Van den Bosche, 1985).

Postharvest treatments were not initially considered as effective in the control of latent CBS infection, since the infection is located inside the rind. However, following the application of IMZ, a substantial amount of the residue (91 – 94%) is translocated into the exocarp (peel) (Brown *et al*., 1983). Further studies conducted by Dore *et al.*, (2009) found that IMZ applied in an aqueous solution has a better curing effect than a protecting effect. Studies conducted by Holmes and Eckert (1999) showed increasing effectiveness of IMZ as the pH ranges from 5.1 to 5.9.

IMZ is available in different formulations that also require different application methods. In research conducted in following chapters, emulsifiable concentrate (EC) in a wax application, and soluble granules (SG) in a dip application were used (Laville *et al*., 1977). For export to EU markets the maximum residue level (MRL) of IMZ on citrus fruit is 5 mg/kg (European Commission, 2016).

In vitro trials conducted by Korf *et al*. (1998) showed that CBS grown on IMZ amended plates (2.12 µl a.i. ml^{-1}) lowered conidial germination to 0,1% and appressorium formation to

0%. Plates amended with imazalil sulphate (2.04 µl a.i. ml^{-1}) reduced conidial germination from 68.8% to 2.0 % and appressorium formation from 55,2% to 0,8%. In his final evaluation of *in vitro* studies, he concluded that the water soluble IMZ sulphate had moderate efficacy, and the emulsifiable IMZ good efficacy against CBS.

Thiabendazole

The first reports involving thiabendazole (TBZ) was as an anthelmintic agent, and it was not until much later that its potential for controlling fungal diseases was recognized (Brown *et al*., 1961; Staron and Allard, 1964). Although useful as a broad spectrum antifungal compound, TBZ is selective in terms of pathogens that are resistant to it, which allows it to be used as a general fungicide on a variety of crops (Allen and Gottlieb, 1970). The fungicide is used extensively in commercial citrus production areas to prevent postharvest decay during storage and shipping periods (Reuther, 1989).

TBZ (2-(4'-thiazolyl) benzimidazole) is a FRAC code 1 fungicide, and belongs to the benzimidazole chemical group (FRAC, 2016). Use of this fungicide is regarded as high risk, since over application or misuse of this chemical can easily result in the pathogen developing resistance to it. In a study conducted by Staron and Allard (1964) they concluded that TBZ has two types of actions attributed to it. Firstly, it inhibits transamination, which is then partially counteracted by exogenous pyridoxine and biotin. Secondly, it interferes with the transfer of amino acids in protein synthesis, which in turn leads to decreased synthesis of various cellular components. In a later study conducted by Allen and Gottlieb (1970), they concluded that the primary site of inhibition is the terminal electron transport system of the mitochondria, leading to secondary decreases in metabolic function. TBZ causes inhibition of spore germination and, should germination still occur, it then causes stunting and malformation of the germ tubes (Allen and Gottlieb, 1970).

TBZ is systemically distributed through the plant tissue, which makes it a more efficient broad spectrum fungicide, which is why it was postulated that it may have potential against latent CBS infections (Staron *et al*., 1966). The most commonly used formulation of TBZ is a suspension concentrate (SC) that is added to water in the drench mixture or to wax. For export to EU markets the MRL for TBZ on citrus fruit is 5 mg/kg (European Commission, 2016).

In vitro studies conducted by Korf *et al*. (1998) showed that CBS cultured on TBZ-amended plates (4,06 µl a.i. ml^{-1}) reduced conidial germination from 64.8% to 0% and appressorium formation from 56.7% to 0%, leading to the conclusion that TBZ was effective in controlling CBS *in vitro*.

Guazatine

First introduced in 1974 in Sweden (Hertfordshine, 2016), guazatine triacetate was used for the control of a variety of soil-borne cereal diseases. In South Africa guazatine (GZT) was the main fungicide used for postharvest control of sour rot of citrus, although it is also registered for use against green and blue mould (Lesar and Erasmus, 2014; ICA International Chemicals, 2016). GZT is a broad spectrum non-systemic contact fungicide, making it useful on a wide range of crops and against different pathogens (Hertfordshire, 2016).

Guazatine triacetate 1,1'iminodi(octamethylene)diguanidine has a FRAC code M7, belonging to the guanidine group (FRAC, 2016). It is regarded as a broad-spectrum, low risk fungicide, without any signs of resistance development (FRAC, 2016). GZT's primary mode of action is to inhibit lipid biosynthesis, with multisite activity that prevents resistance development. The fungicide disrupts membrane structures, decreasing the cellular permeability through a decrease in oxidative capacity. This is accomplished through the inhibition of the uptake of certain substrates, rather than a direct effect on the enzyme activity (Hertfordshire, 2016).

Although previous *in vitro* studies conducted by Korf *et al.* (1998) had very promising results using GZT against latent CBS infection located in the rind, it is primarily a non-systemic contact fungicide, which makes it less likely to be effective. However, the *in vitro* trials conducted by Korf *et al.* (1998) showed reduced conidial germination (62.5% to 0%) and appressorium formation (50.3% to 0%) in CBS cultured on GZT amended plates (1.0 µl a.i. ml^{-1}).

The compound is offered in various types of formulations, but in South Africa the most commonly used was soluble concentrate (SL), which was added to the drench mixture (Lesar and Erasmus, 2014). Up to 2015 the MRL was 5 mg/kg, but in March 2016 the MRL for EU markets was lowered to 0.05 mg/kg on all citrus fruit, which implies that it can no longer be used in packhouses that export to EU markets or markets adhering to EU standards (European Commission, 2016, European Food Safety Authority, 2014).

Pyrimethanil

Pyrimethanil (PYR) is a relatively new chemical in the citrus industry, but was previously used against *Botrytis* spp. on pome fruit (Daniels and Lucas, 1995). The active was first approved in 2005 for use on citrus fruit to control green mould (Smilanick *et al.,* 2006). PYR is utilized both in the orchards and in the packhouse on various crops and is a broad spectrum contact and translaminar fungicide (Krieger, 2001; ICA International Chemicals, 2016).

A FRAC code 9 fungicide, PYR (4,6-dimethyl-N-phenylpyrimidin-2-amine) belongs to the anilino-pyrimidine chemical group (FRAC, 2016). These fungicides are regarded as medium

risk, with no cross-resistance with other chemical groups (FRAC, 2016). The fungicide's mode of action includes inhibition of methionine biosynthesis and secretion of hydrolytic enzymes (Krieger, 2001). The plant cell-wall degrading enzyme secretion system of pathogenic fungi was identified as a target for anilino-pyrimidines. These enzymes play a critical role during the infection process, and in the absence of these enzymes infection cannot take place (Milling and Richardson, 1995). PYRis a contact and translaminar fungicide, used in the citrus industry as a suspension concentrate (SC), which is added to the drench mixture. For all producers exporting to EU markets the MRL for PYR is 8.0 mg/kg (European Commission, 2016). Agostini *et al.,* (2006) previously conducted studies using PYR to control latent infections, but concluded that no application of a single fungicide had any significant inhibiting effect on lesion development.

Chlorine

Chlorine is used in the packhouse wet dump system as a sanitizer for the water used in cleaning fruit from orchard dust and debris before it enters the pack line. Chlorine can also be used in a high pressure washing system on brushes, where it has the same purpose as in the chlorine bath but with the additional mechanical action of the brushes the fruit are cleaned more effectively (Lesar, n.d). There is no maximum residue level set for chlorine in any market (European Commission, 2016).

In previous studies conducted by Korf *et al*. (1998) infected fruit were treated with chlorine at different temperatures and isolations were made from lesions to test viability. Results indicated chlorine treatment had no significant effect on CBS lesion viability.

2,4-D

2,4-D (2,4-dichlorophenoxy acetic acid) is a plant growth regulator and, since the 1950's, has been applied on packlines to retard calyx abscission (Steward *et al.,* 1952). For postharvest purposes 2,4-D is usually added to the drench mixture, applied as a dip, or mixed into the wax and then applied to the fruit. The importance of calyx retention cannot be over emphasized, as the open tissue left once the calyx is abscised is a primary infection route for all postharvest fungal pathogens. Calyx retention reduces the incidence of postharvest decay on the fruit significantly (Dewolfe *et al*., 1959).

The 2,4-D formulation used in the packhouse is a soluble concentrate (SL), which is added to the drench or the wax (FRAC, 2016). The current maximum residue levels for all producers exporting to EU markets is 1,0 mg/kg (European Commission, 2016). There have been no trials conducted on the effect of 2,4-D in terms of controlling latent CBS infection.

This chemical is however included in standard packhouse treatments and was therefore important to research through literature.

Wax

Fruit waxing is a common commercial practice to maintain the freshness of fruit by reducing dehydration, but which, importantly, also improves the appearance of the fruit by delaying aging and increasing resistance to rind damage (Deetlefs, 1959; Long and Leggo, 1959). Wax is also used as a delivery medium for postharvest fungicides that serve as protective measure against postharvest fungi.

The most commonly used commercial waxes contain 14%, 18% or 20% solids in the emulsions, with varying blends of oxidised polyethylene, carnauba and shellac as the functional ingredients (Deetlefs, 1959; Citrashine label). While there is no maximum residue level for the wax itself, any augmenting chemicals being used in the wax do have MRL's and over-application of wax can result in exceeding the allowed MRL of these chemicals.

Research conducted by Seberry *et al*. (1967) followed up on remarks made by Long and Leggo (1959) who stated that wax treatment reduced the development of CBS lesions on fruit. In these trials Seberry confirmed that applying a wax coating to Valencia fruit significantly reduced the expression of CBS lesions of stored fruit.

Alternative fungicides and GRAS chemicals selected for control of latent CBS infections

Propiconazole

Propiconazole (PPZ) was first registered in 1988 for use on seed grass (EPA, 2006). The active was thereafter registered on cereals for control of diseases caused *by Erysiphe graminis, Pseudocerosporella herpotrichoides*, *Puccinia* spp., *Pyrenophora teres*, and *Septoria* spp. (Worthing, 1983; Thomson, 1997). It has mainly been used as a preharvest application on various crops, but has recently been registered on citrus as a postharvest application for use against sour rot (McKay *et al.,* 2012). After the loss of the guazatine MRL in European markets, PPZ is now considered as the most important postharvest fungicide against sour rot (European Commission, 2016; Christie, 2016). Unfortunately, a complicating issue for packhouses is the disallowance of the active in markets like Japan and Gulf Standardisation Organisation (GSO) countries, which means that, due to concerns of cross-contamination by equipment like brushes and rollers, use of the active is not viewed favourably.

As another FRAC code 3 fungicide, PPZ (1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl] methyl] -1H -1,2,4-triazole) belongs to the triazole chemical group (FRAC,

2016). Triazoles are regarded as a medium risk fungicide group with potential for cross-resistance between DMI-fungicides active against the same fungal pathogens (FRAC, 2016). PPZ acts as a ergosterol biosynthesis inhibitor by demethylation of C-14 during ergosterol biosynthesis leading to the accumulation of C-14 methyl sterols, critical for the formation of fungal cell walls (EPA, 2006). The lowering of sterol production slows or stops the growth of fungal mycelia, preventing continued infection of the host tissue (FRAC, 2016; European Commission, 2016; Thomson, 1997). Furthermore, PPZ is a systemic fungicide that has both protective and curative activity (Thomson, 1997, Worthing 1983).

There are two formulations of the active ingredient available: emulsifiable concentrates (EC) and wettable powders (WP) (PMEP, 2016). PPZ is also formulated with other fungicides, with Propirly 270 EC (ICA International Chemicals, Stellenbosch, South Africa) available in South Africa as such a blended formulation. Propirly 270 EC (ICA International Chemicals, Stellenbosch, South Africa) is a EC formulation containing both PPZ and PYR. The PPZ MRL for EU markets varies depending on citrus cultivars. The MRL for grapefruit, lemons, limes and mandarins is 5 mg/kg; and the MRL for oranges is 9 mg/kg (European Commission, 2016). To date, no previous studies have been done to determine whether PPZ is effective against citrus black spot.

Fludioxonil

Fludioxonil (FLU) a synthetic analogue of the bacterial metabolite pyrrolnitrin (Schirra *et al*., 2005; Zhang and Timmer, 2007). The active was first registered for postharvest use in America in 2003, and in 2005 it was tested as an alternative to IMZ for use in *Penicillium* control (Schirra *et al*., 2005; Förster *et al*., 2007).

FLU (4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile)) is a FRAC code 12 fungicide and belongs to the chemical group phenylpyrroles (FRAC, 2016). It has been found to be effective as a postharvest treatment to control a broad spectrum of fungal pathogens. The mode of action is not yet fully understood, but to date it is known that FLU inhibits transport-associated phosphorylation of glucose, prevents glycerol synthesis (FRAC, 2016; Abad-Fuentes *et al.,* 2014). The use of FLU consequently leads to inhibition of mycelium growth, also lowering osmotic signal transduction, inhibiting spore germination, and germ tube elongation (Hertfordshire, 2016; Rosslenbroich and Stuebler, 2000).

As a broad spectrum, non-systemic fungicide, FLU has a long residual activity (Hertfordshire, 2016). The formulation used in the industry is a suspension concentrate (SC). In research trials conducted, in the current study, FLU was mixed into water and applied as a dip treatment. The MRL for all citrus varieties is 10 mg/kg (European Commission, 2016). FLU has been used in black spot trials conducted by Agostini *et al*. (2006), but these studies

indicated that no single fungicide application had any measurable effect on inhibiting lesion formation.

Potassium sorbate

Potassium sorbate is used in citrus packhouses to protect fruit against postharvest decay. Previously, the main use for sorbates was limited to maintaining the quality of raw fruit and vegetables. Sorbic acid and potassium sorbate are still used in food preservation worldwide (Chichester and Tanner, 1968). Potassium sorbate is classified as a GRAS chemical (*Generally Regarded as Safe* for use in food products) by the U.S Food and Drug Administration, but unfortunately its use is not allowed on any food exported to the EU (European Commission, 2016).

Sorbate inhibits the growth of a broad spectrum of microorganisms by effectively inhibiting spore germination in various classes of microorganisms. There is however a possibility for resistance development (Mendonca, 1992). Resistance to sorbate is dependent on the concentration of sorbate present, and the ability of the microorganism to metabolize the sorbate (Mendonca, 1992). The precise mode of action for potassium sorbate is not well understood and further studies are still required (Sofos and Busta, 1981).

In this research study potassium sorbate was used as a wettable powder (WP) that was suspended in water, and applied as a dip treatment to the fruit. There have been no previous studies conducted evaluating the effect of potassium sorbate on latent citrus blackspot infections.

Sodium bicarbonate

Sodium carbonate (Na_2CO_3) has been used as an antimicrobial agent for a long time. Studies conducted by Palou *et al.* (2001) found sodium bicarbonate to be effective in controlling green mould on fruit stored at 20°C, but control proved to be inferior during long periods of cold storage. Studies conducted by Erasmus *et al*. (2015) found that adding sodium bicarbonate to the IMZ fungicide bath increased the pH and subsequent increased IMZ residue loading.

The GRAS classification of sodium bicarbonate resulted in its acceptance for use in all food products (Lakhanisky, 2012). It can therefore be used on all fruit exported to the EU, with no MRL requirement (European Commission, 2016). In our trials, we used a wettable powder that was dissolved in water, and the solution then used for a dip treatment. Prior the trials in this study there was no literature indicating that sodium bicarbonate has previously been used to control citrus blackspot infection.

AIM OF THIS PROJECT

From all available literature, the consensus is that CBS is not a postharvest disease. However, it is a postharvest problem since the latent infections can express postharvest symptoms. Citrus fruit produced for fresh domestic and export markets are subjected to standard postharvest handling and treatment programmes. These programmes consist of a fungicide drench application, a chlorine or sanitiser treatment, the fungicide application in spray, dip or wax application and cold storage (Lesar and Erasmus, 2014). The program is specifically designed to maintain fruit quality and to protect fruit against the main postharvest diseases such as green mould and sour rot. Postharvest handling and treatment have been showed to have variable effects on the viability of latent CBS infections, lesions and spores produced (Korf, 1998; Agostini *et a*l., 2006; Seberry *et al*, 1967). Since these studies, the specific products as well as handling and application regimes have changed to improve postharvest quality and disease control, which might also affect the concomitant effects on *P. citricarpa* in latent or visible infections.

The objectives in this research study were to evaluate current citrus postharvest treatments being used in South Africa, as well as alternative chemicals. These include:

1. Investigating the singular and combined effects of standard postharvest fungicide treatments and cold storage regimes on the viability and reproductive ability of CBS lesions;
2. Investigating the effects of new and alternative fungicides, as well as non-fungicide compounds, applied in heated aqueous applications on the viability and reproductive ability of CBS lesions.

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FIGURES

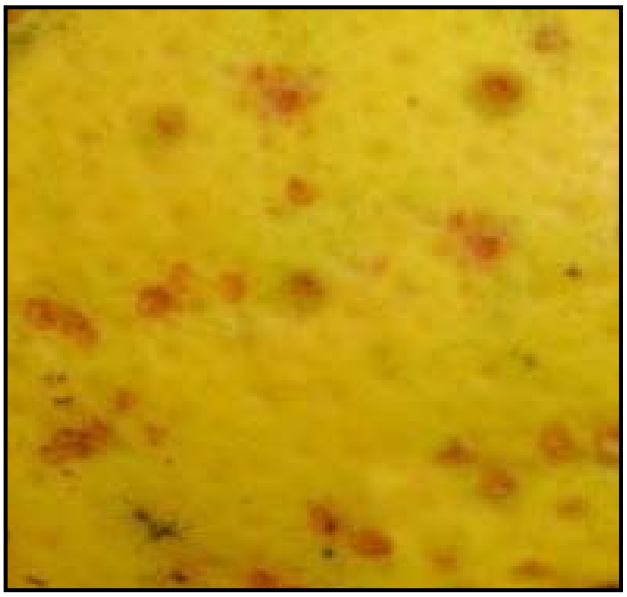

Figure 1: *Phyllosticta citricarpa* red spots on Eureka lemons (Photo courtesy of Elaine Basson, CRI Nelspruit)

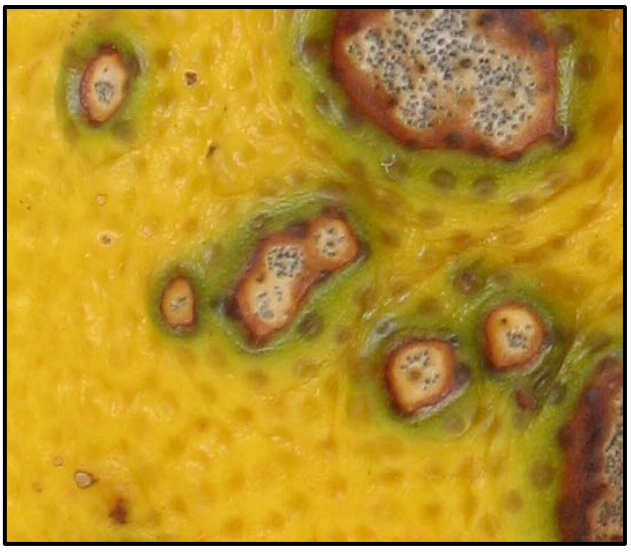

Figure 2: Hard spot symptoms on fruit. (Photo Courtesy of Tian Schutte, CRI, Nelspruit)

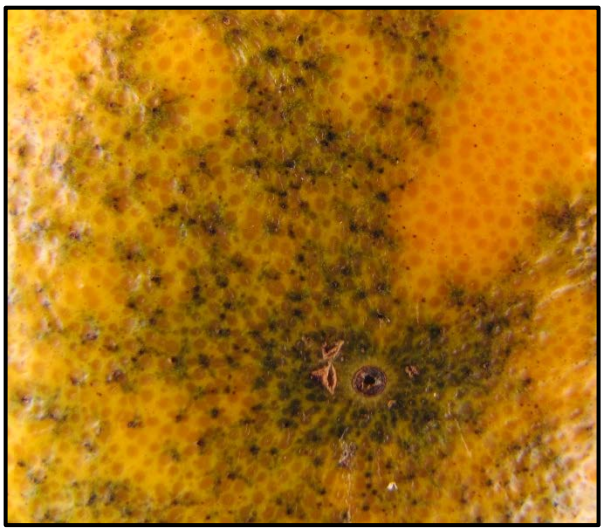

Figure 3: False melanose symptoms on fruit. (Photo courtesy of Elaine Basson, CRI, Nelspruit)

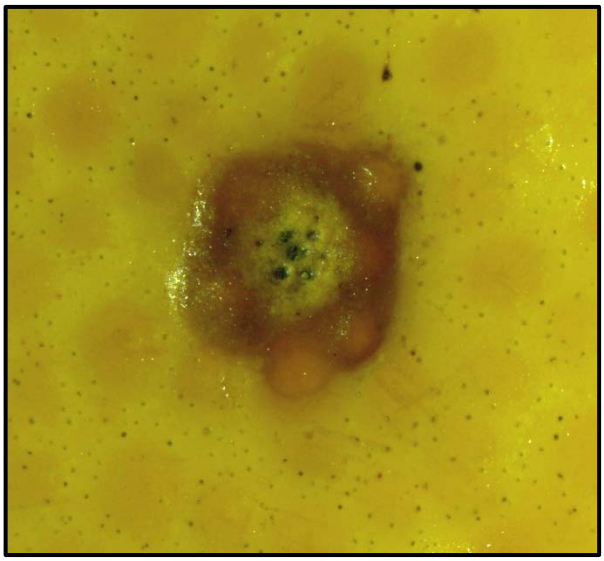

Figure 4: Virulent spot on fruit. (Photo courtesy of Elaine Basson, CRI, Nelspruit)

Figure 5: *Phyllosticta citricarpa* Cracked spot on eureka lemons (Photo taken by Wouter Schreuder, CRI, Nelspruit)

CHAPTER 2

Investigating the singular and combined effects of standard postharvest fungicide treatments and cold storage regimes on the viability and reproductive capability of citrus black spot lesions

ABSTRACT

Phyllosticta citricarpa is the causal agent of citrus black spot (CBS), and is regarded as a quarantine organism. Some South African export markets, such as the European Union, have imposed very limiting trade restrictions for fresh fruit exported from production areas where this organism occurs. CBS is effectively controlled using timely applied preharvest fungicide sprays. However, fruit infections that were not prevented often remain latent until after the asymptomatic fruit is harvested, treated and packed in packhouses and exported. The objective of this study was to evaluate the efficacy of postharvest treatments for the control of latent CBS infections, and determine the reproductive ability of lesions that formed after these treatments. Trials were conducted on naturally infected fruit, harvested from four Eureka lemon and four Valencia orange orchards. Fruit with CBS lesions, as well as asymptomatic fruit with latent infections, were subjected to standard packhouse sanitation and fungicide treatments, cold storage (singularly and combined), subjected to a 5-week cold or ambient storage and incubated for 2 weeks at conditions conducive to expression of latent infections. The pre-packhouse drench and chlorine wash had no significant effect on control of latent infections. On the other hand, imazalil (IMZ) dip treatments at 25 to 55°C mostly resulted in moderate to significant control of latent infection. Although there was no direct correlation to temperature of application, IMZ dips appear to be more effective on Valencia oranges. Wax application to fruit also resulted in moderate to significant control of latent infections, but more importantly, during viability studies it was observed that lesions on fruit treated with wax did not release pycnidiospores from pycnidia that formed in new lesions. The full packhouse treatment, along with a cold storage period (Valencia oranges 4°C, Eureka lemons 7°C), consistently effected significant control. Viability trials conducted on newly formed lesions proved that fruit lesions have a very low reproductive capability with 0.001-2.09% new lesions on Eureka lemons, and 0-0.35% on Valencia oranges forming pycnidia. Trials conducted on fruit with visible lesions indicated that post-harvest treatments had no consistent effect on lesion viability. The combined epidemiological requirements for pycnidiospore release, along with results from trials evaluating postharvest treatment effects on latent CBS infections, indicate that harvested fruit is not an epidemiologically significant pathway for the spread of CBS.

INTRODUCTION

South Africa is the second largest exporter of fresh citrus fruit worldwide, with exports accounting for approximately a quarter of the global market (CGA, 2016). In 2015, 40% of citrus exports went to European markets (CGA, 2016). Maintaining access to these export markets requires adherence to the specific quality standards that have been imposed (European Commission, 2000).

CBS caused by *Phyllosticta citricarpa* (McAlpine) van der Aa, is a cosmetic disease of citrus fruit. The disease remains mostly latent in infected fruit and can express a variety of black spot lesions on rinds of maturing fruit. These lesions are not progressive postharvest decays, but makes fruit aesthetically unfit for the fresh fruit market (Kotzé, 1981). In severe cases, and in highly suitable climates, heavy fruit infection can also lead to premature fruit drop and crop loss (Araújo *et al.*, 2013). In South Africa, CBS rarely causes crop loss, but it is an economically important disease. *Phyllosticta citricarpa* is regarded as a quarantine pathogen in certain countries and the presence of CBS in production regions or the presence of CBS lesions on fruit limit access to those markets (Carstens *et al*., 2012). Current EU regulations, for example, allow citrus fruit to be exported only if produced in pest-free zones of production, or orchards that were free of the disease. Whole consignments will be rejected if one or more CBS lesions are observed upon inspection of fruit (Anonymous, 2000). The EU's zero tolerance for CBS has been challenged by South Africa (South African CBS PRA, 2000-2009), and the EU's recent pest risk assessment for *P. citricarpa* (EFSA, 2014) was criticised by an international panel of CBS experts (CBS Expert Panel, 2013; 2014; 2015). The CBS Expert Panel (2013, 2014, 2015) agreed with earlier pest risk assessments, conducted by South Africa and USA (USDA-APHIS, 2010), in which it was concluded that fruit is not a realistic pathway for CBS to enter, establish, or spread and have significant economic impact within the EU.

CBS is primarily controlled in the orchard through the application of curative and/or protective fungicidal sprays to protect young, susceptible fruit during the fruit susceptibility period (the first 4 to 5 months after petal drop) (Kiely, 1948; Schutte, 2002; Schutte *et al.*, 2003; Miles *et al.*, 2004). Such a preharvest spray program generally consists of 3 to 4 applications, combining and alternating between mancozeb, benzimidazoles, strobilurins and copper compounds (Kellerman and Kotzé, 1977; Schutte *et al.,* 2003; Miles *et al.,* 2004). Whilst very high levels of control can be achieved (Makowski *et al*., 2014), even timeously applied fungicides can result in variable protection due to climatic conditions, coverage of the fruit surface achieved by the spray, and cultivar susceptibility (Calavan, 1960; Kiely, 1969, 1970, 1971; Schutte, 2002; Schutte *et al.*, 2003).

CBS disease control measures are very effective, but fruit with latent infections can remain asymptomatic during picking and packing, and might subsequently display lesion expression during shipping (Kiely, 1948; Loest, 1958; Brodrick, 1969). The export of fresh citrus from affected production areas is therefore becoming increasingly difficult, particularly to the EU markets with a zero tolerance for CBS. Furthermore, unacceptable levels of non-compliance might lead to closure of the market (WTO, 1993; Anonymous, 2000).

South African fresh fruit has some of the longest export routes. From the time of harvest until the fruit reaches the consumer, can take between 6 to 10 weeks, depending on the export country (Pelser, 1977). Due to these long periods, producers and packhouses are required to apply effective control measures, such as postharvest fungicides, wax coatings and sanitation practices to maintain product quality and limit postharvest decay. Asymptomatic fruit with latent *P. citricarpa* infections, as well as fruit with CBS lesions, will be subjected to these standard packhouse handling and treatment processes. It is therefore important to evaluate the effects of these treatments on *P. citricarpa* infection and expression on the harvested fruit.

The first published studies evaluating the effect of postharvest fungicides on CBS were conducted by Korf in 1998. During these trials, Korf (1998) conducted *in vitro* studies which showed postharvest fungicides imazalil (IMZ), thiabendazole (TBZ), prochloraz, guazatine (GZT) and sodium ortho-phenylphenate (SOPP) significantly reduced conidial germination and appressorium formation of *P. citricarpa*. Korf also conducted *in vivo* trials using chlorine, and found that a chlorine treatment had no significant effect on CBS infections. Previous work on the effect of postharvest fruit treatments gave variable results on the viability of *P. citricarpa* lesions varies (Korf *et al*., 2001; Agostini *et al*., 2006). Korf *et al*. (2001), in addition, demonstrated a 3- to 7-fold reduction in *P. citricarpa* viability. The effects of fungicide treatments on the reproductive ability of *P. citricarpa* (latent or visible) have never been investigated, neither has modern packhouse treatments been evaluated. Agostini *et al.* (2006) conducted fruit trials using different postharvest fungicides, and reported that none of the fungicides had a significant effect on lowering postharvest CBS incidence. Instead, storing fruit at 8°C significantly reduced postharvest incidence of CBS. Seberry *et al.* (1967) reported that applying a wax coating to Valencia fruit significantly reduced the expression of CBS lesions of stored fruit.

The aim of this study was to evaluate the effect of current postharvest fungicides and application methods being used in South African citrus packhouses on latent *P. citricarpa* infection, focussing on viability and reproductive capability of the pathogen.

MATERIALS AND METHODS

Fruit used in trials

Eureka lemons and Valencia oranges were used in all the trials, and were chosen for their high susceptibility to *P. citricarpa* (Kiely, 1948). Fruit were harvested from farms located in Gauteng, Mpumalanga and Eastern Cape provinces. Lesion-free fruit were collected from unsprayed or abandoned orchards that were verified to have CBS infection following prior inspection. Two thousand fruit were harvested into plastic fruit crates (325 x 505 x 245 mm) and transported to CRI facility located in Nelspruit, Mpumalanga. Eight different orchards were used: four Eureka lemon and four Valencia orange orchards.

Postharvest treatments

Individual treatments of the pre-packhouse drench, chlorine wash, wax and IMZ dips (applied at 25°C, 35°C, 45°C and 55°C) were evaluated as single treatments. The full packhouse treatment consisted of the pre-packhouse drench, chlorine wash, IMZ dip at 35°C, and a wax application, and was evaluated as a combination treatment.

Pre-packhouse drench

Fruit were drenched before degreening to minimize postharvest decay, primarily caused by *Penicillium* spp. and *Galactomyces* spp. (Ladaniya, 2010). The drench mixture of TBZ (1000 $mg.L^{-1}$), PYR (1000 $mg.L^{-1}$), GZT (1000 $mg.L^{-1}$) and 2,4-D (250 $mg.L^{-1}$) was prepared by filling a container with 50 L of tap water and adding fungicides in the order that follows, while constantly stirring the mixture to keep all the fungicides in suspension: 100 mL thiabendazole (Thiabendazole, 500 $g.L^{-1}$ SC, ICA International Chemicals, Stellenbosch, South Africa), 125 mL PYR (Protector, 400 $g.L^{-1}$ SC, ICA International Chemicals, Stellenbosch, South Africa), 240 mL GZT (CitriCure, 210 $g.L^{-1}$ SL, ICA International Chemicals, Stellenbosch, South Africa) and 500 mL 2,4-D (2,4-dichlorophenoxy acetic acid) (Deccomone, 25 $g.L^{-1}$ SL, Citrashine, Johannesburg, South Africa).

In these trials, a simulated dip-drench system was used (Christie, 2016). Fruit were packed into plastic crates (dimensions: 325 x 505 x 245 mm) which were used to simulate the standard 800 L orchard bin used by commercial citrus packhouses. The drench solution was applied at a rate of 12.5 L/min per crate (Christie, 2016) at an exposure time of 1 minute (a dosage similar to commercial drench applicators), and then left to dry for 24 hours before other treatments were applied, before incubation.

Chlorine wash/dip

The chlorine wash system is generally a wet dump flume system where the fruit first enters the packhouse after degreening, or alternatively (if not degreened) directly from the orchard, depending on the producing unit's operations. The main purpose of the wash system is to remove spores, organic material, and dirt that may have collected on the fruit in the orchard, and during picking and transport (Lesar, n.d.). Chlorine acts as a sanitiser to reduce the build-up of fungal and bacterial spores in the wash water, preventing it from becoming an inoculum source.

The wash solution was prepared by adding 15 g of calcium hypochlorite (HTH Pool Chlorine, 680 g.kg $^{-1}$ WP) to 50 L of tap water to give a 200 mg.L^{-1} total chlorine solution (CRI recommendation sheets, 2015). Hydrochloric acid was used to adjust the pH to 6.5-7.5 and confirmed by pH meter (Jenway Model 3310; Bibby Scientific Limited, Staffordshire, UK). To simulate the wet dump sanitation system, fruit were submerged in this solution for 90 seconds and then left to dry.

Fungicide bath

The specific purpose of the IMZ dip tank is control of green mould (*Penicillium digitatum*) (Harding, 1976), with the commonly used operating temperature of 35°C (Erasmus *et al.,* 2011, 2013). In this study, treatments were applied at 25°C, 35°C, 45°C and 55°C to evaluate how temperature would affect treatment efficacy.

An IMZ solution was prepared by dissolving 80.4 g of imazalil sulphate (Imazacure, 750 g.kg^{-1} SG, ICA International Chemicals, Stellenbosch, South Africa) in 120 L of tap water to give a 500 mg.L^{-1} solution. The pH was constantly measured with a pH meter (Jenway Model 3310; Bibby Scientific Limited, Staffordshire, UK) and kept between 3.5 – 4 by adding hydrochloric acid. This was done to keep residue loading constant at different application temperatures (Erasmus *et al.,* 2011, 2013). Citrus fruit were submerged in the solution for 60 seconds and then put over revolving brushes, as is the common practice in packhouses. The equipment used for the dip application was an experimental packline developed and custom built to simulate commercial packlines.

Wax application

A smaller version of a commercial waxing unit (Decco Citrashine (Pty) Ltd, South Africa, Johannesburg) was used for application of a polyethylene-based wax (PolyOrange, 18% solids, Decco Citrashine (Pty) Ltd, Johannesburg, South Africa). The wax suspension consisting of IMZ (2000 mg.L^{-1}), TBZ (4000 mg.L^{-1}) and 2,4-D (250 mg.L^{-1}) was prepared by pouring 25 L of wax into the mixing tank and adding the treatment chemicals to the wax while

the pump was constantly mixing the solution. The individual fungicides were first dissolved in 100 mL of warm water (40°C), before adding them to the mixing tank in the following order: 100 mL of IMZ (Imazacure, 500 $g.L^{-1}$ EC, ICA International Chemicals, Stellenbosch, South Africa), 200 mL of TBZ (Thiabendazole, 500 $g.L^{-1}$ SC, ICA International Chemicals, Stellenbosch, South Africa) and 250 mL 2,4-D (2,4-dichlorophenoxy acetic acid) (Deccomone, 25 $g.L^{-1}$ SL, Decco Citrashine (Pty) Ltd, Johannesburg, South Africa).

The wax applicator tank was filled and left to agitate for 15 min before treatments commenced. The wax brushes were optimally wetted (damp to the touch) before use, and the wax applicator calibrated to administer 1 - 1.2 L of wax per ton of fruit.

Cold storage

Valencia oranges and Eureka lemons were placed into cold storage as a single treatment, and as part of the combination treatment. Eureka lemons are more prone to cold damage at low temperatures, therefore lemons were stored at 7°C and Valencias, less prone to cold damage, were stored at 4°C for 5 weeks. Valencias used in cold sterilization trials were stored at -0.5°C for 24 days, then moved to cold storage (4°C) for remaining 11 days, as per industry recommendations.

Residue analysis

Six fruit were sampled from replication one and replication four, after each treatment application. The sampled fruit were placed in residue bags and deep frozen (-20°C), then later used to prepare pulp for residue analysis. The fruit inside residue bags were defrosted, measured and weighed. The fruit were then chopped and macerated to a fine pulp using a blender (Salton Elite, Amalgamated Appliance Holdings Limited, Reuven, South Africa) and the pulp was then re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (detected as chloramizole), PYR (detected as pyrimethanil), TBZ (detected as thiabendazole) and 2,4-D (detected as the free acid) residue analyses by Hearshaw and Kinnes Analytical Laboratory (Cape Town, South Africa). The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA). The data collected was analised and the range at which fungicide residues were loaded was determined to confirm that no maximum residue limits (MRL) were exceeded (SAS Institute Inc. Cary, NC, USA).

Trials and evaluations

The trials that were conducted consisted of four replicates per treatment, with each replicate consisting of 12 fruit. For residue analysis, six extra fruit was included in the first and fourth replicates of each treatment.

Effects of treatments on latent infections

Latent infection trials were conducted four times each on Eureka lemons and Valencia oranges. Treatment protocols were as follows:

- Individual applications of the pre-packhouse drench, chlorine wash, wax and IMZ dips (applied at 25°C, 35°C, 45°C and 55°C) and cold storage (5 weeks at 7°C for lemons or 4°C for oranges).
- The full packhouse treatment consisted of the pre-packhouse drench, chlorine wash, IMZ dip at 35°C, and a wax application, and evaluated as the combination treatment. In this case, the pre-packhouse drench application was applied 24 hours before any of the other treatments to simulate the practices followed in a commercial packhouse. The full packhouse treatment was done in duplicate, with and without cold storage.

After treatments were applied, fruit were packed into lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa). Each carton was covered with a transparent polyethylene bag and sealed with a cable tie to ensure that a high relative humidity atmosphere was maintained.

The single treatments and one of the full packhouse treatments were stored at room temperature (22°C, 79-85% RH) for 5 weeks, along with an untreated control. A duplicate of the full packhouse treatment was placed into cold storage for 5 weeks, also along with individual cold storage treatment.

Effects of different cold storage regimes on latent infections

The effect of the full packhouse treatment, followed by storing *P. citricarpa* infected fruit at different cold storage regimes, was evaluated. The trials were conducted three times on Valencia oranges. The full packhouse treatment was done in triplicate. Fruit from one full packhouse treatment were stored at ambient conditions along with untreated control fruit for 5 weeks, fruit from another full packhouse treatment was placed into cold storage (4°C) for 5 weeks along with untreated control fruit, and fruit from the third full packhouse treatment went into cold sterilization (-0.5°C) for 24 days along with untreated control fruit, where after the fruit from cold sterilization were moved to cold storage (4°C) for another 11 days. After 5 weeks,

fruit were moved from the storage areas into the incubation room, for incubation and rating as described below.

Effects of treatments on symptomatic fruit

The effects of postharvest fungicide treatments on fruit with CBS lesions were also evaluated. In these trials, Eureka lemon fruit with visible hard spot lesions were used. Single and combination treatments were applied to fruit with lesions, as described above, except for combinations treatments with cold storage.

After 2 weeks, six fruit from each replicate were used to determine reproductive potential of treated lesions. From the remaining six fruit in each replicate, four random fruit were chosen and then four random lesions per fruit were isolated, to determine viability. A total of 96 lesions were isolated per treatment. The selected lesions were marked and the fruit submerged in 70% ethanol for 1 min, then placed on sterilized tissue paper inside the laminar flow to dry. Once the fruit had dried the marked lesions were isolated onto Potato-Malt-Yeast agar (PMY agar) plates (Korf, 1998). PMY agar consists of 24 g potato-dextrose agar (PDA; DifcoTM; Becton, Dickinson and company; Sparks; MD; USA), 1 g yeast extract (YE; Biolab, Merck, Gauteng, RSA), 1 g malt extract (ME; Biolab, Merck, Gauteng, RSA) and 8 g agar (DifcoTM; Becton, Dickinson and company; Sparks; MD; USA), hydrated and sterilised in 1 L of deionised water. Lesion viability was evaluated as described below.

Isolations were made from lesions by first cutting a small square (1 cm^2) around the lesion. Using the scalpel, a thin layer consisting of the albedo and bottom of the lesion was removed and discarded. With the bulk of the lesion and some flavedo being exposed, an approximately 250 µm cross-section of the lesion and surrounding tissue was removed. This cross-section was then cut into four equal quarters, with the lesion in the centre. The four quarters were then plated out onto PMY agar and the rest of the lesion was removed and placed into 1.5 mL Eppendorf tube to be used for PCR identification. The plates were placed in an incubator (24-hour light, 25°C and RH> 85%), left for 10 to 14 days and then inspected.

Each plate represented a single lesion, therefore if there was mycelial growth from any of the four cross section pieces, the fungal growth in the lesion was considered as still being viable. The data was used to determine the percentage lesions still alive, and how the different postharvest treatments affected lesion viability. Fungal identity was confirmed using the typical growth characteristics of the fungus on oatmeal agar (OA, DifcoTM; Becton, Dickinson and company; Sparks; MD; USA) and PCR.

Evaluation

Lesion expression and reproductive ability

After 5 weeks, fruit stored at cold or ambient conditions were moved to an incubation room. The incubation room was optimised to enhance lesion expression, with the temperature of 25-27°C, 24-hour lighting and RH > 85%, whilst RH inside the bags were near-100%. The option to apply ethepon to accelerate lesion expression (Schutte and Beeton, 1999; Baldassari et al., 2007) was not considered as it was imperative that conditions in a commercial supply chain be simulated. The fruit were incubated for 2 weeks and evaluated for lesion expression.

The CBS lesions on each fruit were counted on day 0 (day when removed from cold or ambient storage), day 7 and day 14, after having been placed in the incubation room. The number of lesions that developed pycnidia was also recorded. After the last count was completed, all fruit with lesions that did not form pycnidia were discarded. Fruit with lesions that formed pycnidia were used in the further determination of the reproductive potential of lesions.

Pycnidiospore release and spore viability

Pycnidiospore release was induced using a solution made of Valencia juice and citric acid. This solution was prepared by adding 50 mL of a 5% citric acid solution to 100 mL of 100% freshly squeezed Valencia orange juice. Using a pipet (Pipetman®, Gilson, S.A.S, Villiers, France-le-Bel) this solution was pipetted into 2 mL Eppendorf tubes and centrifuged for 5 min at 16 000 rpm the supernatant pipetted into 5 mL glass vials and autoclaved for 15 min at 121°C.

Within each treatment, five pieces of fruit with lesions with visible pycnidia were selected. On each fruit, one lesion was marked and cleaned superficially using an earbud dipped in 70% ethanol, and then left to dry in the laminar flow. Once dry, a 5 µL drop of the prepared Valencia juice and citric acid solution was placed on the lesion, using a 20 µL pipette (Pipetman®, Gilson, S.A.S, Villiers, France-le-Bel). The droplet was left on the lesion for 30 minutes, allowing enough time for spores to be released into the droplet (Korf, 1998), without the droplet drying out. Using a pipette, the droplet was collected and the lesion was washed five times, every time using a new 5 µL droplet of sterilized water, to collect as many suspended pycnidiospores as possible. All the liquid collected from the lesion and plated onto a water-agar plate (WA; DifcoTM; Becton, Dickinson and company; Sparks; MD; USA) and incubated in a laminar flow for 24 hours at 22°C, allowing the droplets on the agar surface to dry. After the drying, the plate was examined under a stereo microscope (Zeiss Stemi DV4, Carl Zeiss (Pty) Ltd, Germany) and five pycnidiospores were single-spored onto oatmeal agar plates (OA, DifcoTM; Becton, Dickinson and company; Sparks; MD; USA). The OA plates were

placed in an incubator (24-hour light, 25°C and RH>85%) and left for 10 to 14 days. Oatmeal agar is a selective medium for *P. citricarpa* and *Phyllosticta* mycelium growth with a yellow halo around the colony will indicate that the harvested pycnidiospores were viable, and positively identify it as *P. citricarpa* (Truter, 2010; Anonymous, 2014). If *Phyllosticta* mycelium growth is present but no halo has formed, it is indicative of the non-pathogenic endophyte *P. capitalensis*.

Verification of lesion diagnosis

As per the methods described by Hu *et al.* (2013), real-time polymerase chain reaction (qPCR) was used to verify the morphological identification *P. citricarpa* from the subsamples for each trial.

DNA was extracted from the CBS lesions using the PROMEGA Wizard ® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The manufacturer's instructions were slightly modified as lesion size was typically small. Pellets were re-suspended in 25 µl DNA rehydration solution. Control samples were included in the DNA extraction, including a positive fruit control (lesions from fruit that previously tested positive for *P. citricarpa*) and negative fruit control (fruit with no lesions obtained from a disease-free orchard). The elution volume was reduced to 1 × 75 µL (Meyer, 2006). The extractions were placed in a 1.0% agarose gel with TBE buffer.

Species-specific real-time primers were used to amplify the ITS-region of *P. citricarpa*. The primer pair, GCITSF (5'- CCT GAA AGG TGA TGG AAG GG - 3') and GCITSR (5'- CGC CAA AGC AAC ATG GTA GAT A- 3') (Hu *et al*. 2013) was used in a 20 µl reaction to amplify the region. The qPCR reaction consisted of 2x KAPA Probe Fast qPCR master mix (KAPA Biosystems, Wilmington, MA, USA), 400 nm forward and reverse primer, 200 nm probe and 6 µl of PCR grade water. The thermal cycle consisted of an initial activation step of 95°C for 3 min, then 40 cycles of 95°C for 15 s and 59°C for 30 s. Control samples were a previously positive PCR sample as positive control as well as a no-template control.

Statistical analysis

The counts of the newly formed lesions were used to calculate the percentage control of individual replicates for each treatment relative to the mean lesion expression in the untreated control of each trial. One iteration of the Grubbs lower-tailed test at 5% significance level was performed on percentage control data to identify outliers, which were removed. The counts of lesions containing pycnidia were expressed as mean percentage pycnidia-forming lesions for each replicate in a treatment, within each trial. Data were subjected to appropriate analyses of variance (ANOVA). Fisher's LSD was calculated to identify significant differences between

treatments, using a confidence interval of 90%. All statistical analyses were done using statistical analysis software Addinsoft XLSTAT Version 2015.8.18 (www.xlstat.com).

RESULTS

Effects of postharvest treatments on latent infections present on Eureka lemons

During the first 14 days, an increase in average lesion number expressed per Eureka lemon fruit was observed (day 0 after treatment storage and start of incubation period = 5.9; day 7 = 13.8; day 14 = 31.03 average lesions per fruit). The climatic conditions in the incubation room was perfect for lesion expression, and for the development of postharvest fruit rotting fungi. Day 7 data were regarded as more reliable since fruits had rotten due to postharvest decay by day 14 (mostly green mould and *Phytophthora* brown rot) and resulted in missing data points making data set less reliable. Trial one (3.74 lesions per fruit) and two (7.10) had significant lower average number of lesions expressed than trial three (38.97) and four (78.59).

ANOVA of percentage control data indicated significant effects for trials ($P = 0.001$), for treatments ($P < 0.0001$) and no significant interaction between these factors ($P = 0.045$). The lower disease expression in the control fruit of trial two led to negative percentage values in some treatments, and the mean percentage control in this trial (-35.7%) differed significantly from the others three trials (20.8-53.8%). Table 1 shows the mean percentage control observed following various treatments in the four trials conducted on Eureka lemons. The treatment efficacy can be summarised as follows: no significant control with control levels ranging from -5 to 19% (Pre-packhouse drench, Chlorine wash, IMZ Dip at 35); low levels of control (20-39%; Cold storage, IMZ Dip at 45°C); moderate levels of control (40-59%; Wax, IMZ Dip at 25 and 55°C, Combination Ambient); and significant levels of control (60-100%; Combination with cold storage (7°C)). Individual treatments generally gave variable levels of control, whilst control by the combination treatments was more consistent, as is evident from the standard deviance.

ANOVA of percentages of newly formed lesions that developed pycnidia on Eureka lemons indicated significant differences between trials ($P < 0.0001$), but not between treatments ($P = 0.180$). Trial 1 had significantly more reproductive lesions (1.87%) compared with the other trials (0.05-0.08%). Less than 2.1% of newly formed lesions developed pycnidia. Except for the IMZ dip at 25°C (2.1%), most individual (0.034-0.567%) and combination (0.001-0.320%) treatments had markedly lower percentages of reproductive lesions than the control treatment (1.055%) (Table 1). The counts for lesions with pycnidia on day 14 was generally lower than that on day 7 due to rotten fruit that had to be discarded; data were not analysed further).

Effects of postharvest treatments on latent infections present on Valencia oranges

Ratings were conducted over a period of 14 days; during this period an increase in average lesions expressed per Valencia orange fruit was observed (day 0 = 0.146; day 7 = 0.348; day 14 = 0.542 average lesions per fruit). It was evident from these results that the disease pressure on Valencia oranges was markedly lower than that observed on Eureka lemons. Mean number of lesions in the control treatments ranged from 0.44 to 1.29. The climatic conditions in the incubation room accelerated the development of postharvest fruit rotting fungi. The results from day 7 and day 14, shown in Table 2, have the same trend in terms of percentage control of latent infection. The raw data collected on day 7 were used for statistical analysis because by day 14 too many fruits had rotten and resulted in large quantities of missing data points.

ANOVA of percentage control data indicated significant effects for trials ($P < 0.0001$), for treatments ($P < 0.0001$) and a significant interaction between these factors ($P = 0.0002$). The interaction was ascribed to variable results of individual treatments, and the combined results for the trials are presented. Significantly higher levels of control were observed in trials 3 and 4 (means of 75.2 and 70.0%, respectively), than trial 2 (47.1%) and trial 1 (24.5%). Table 2 shows the mean percentage control observed following various treatments in the four trials conducted on Valencia oranges. The treatment efficacy can be summarised as follows: no significant control with levels ranging from 0 to19% (Drench, Cold storage); moderate levels of control (40-59%; Chlorine wash, Combination 1 (Ambient)); and significant levels of control (60-100%; Combination 1 with cold storage (at 4°C), Wax, IMZ Dip at all temperatures).

The standard deviance indicated the variability in control for some individual treatments (cold storage, drench) and more consistent control for IMZ dip treatments, wax and combination treatments. No significant difference was observed between IMZ dips at the different temperatures. Both the combination treatments (at ambient or cold storage) consistently resulted in significant levels of CBS control.

ANOVA of percentage data of reproductive lesions indicated no significant effects between trials ($P = 0.388$), a significant treatment effect ($P = 0.051$) and a significant interaction between these factors ($P = 0.028$). The trial effect was attributed to variable results for the individual treatments and the mean treatment effects are reported. Table 2 shows the mean percentage of newly formed lesions that developed pycnidia on Valencia oranges as rated on day 7; similar to lemons, the numbers of pycnidia rated on day 14 was similar or reduced, due to the rotten fruit not being rated. Less than 0.17% of newly formed lesions developed pycnidia, and were in most cases similar to the untreated control (0.06%). Pycnidia formation completely inhibited on fruit that received the combination treatments (at ambient or cold storage).

Effect of postharvest treatments on symptomatic fruit

ANOVA of the mean percentage viable fungi in preformed hard spot lesions indicated that no significant difference between treatments (P = 0.412) existed. Lesion viability ranged from 79.2 to 100% and none of the treatments differed significantly (P = 0.412) from the control treatment (79.2%) (results not shown). Results were variable, which was attributed to the manual nature of the isolation technique, contamination of plates by co-isolated saprophytes, and age differences of isolated lesions. Isolation of the fungus from hard spots were more difficult to achieve when fruit were treated with wax. However, these lesions act as the perfect point of entry for saprophytes and secondary pathogens, which was evident on the PMY agar plates. Treating lesions with ethanol did not kill the mycelium of saprophytes present inside the lesion.

Effects of different cold storage regimes on latent infections

Due to postharvest rotting observed in previous trials data for the day 7 rating were used for statistical analysis. Mean number of lesions in the control treatments ranged from 1.02 to 8.05.

Table 3 shows results for percentage control of latent infection present on fruit treated with the combination treatment and different storage regimes. ANOVA indicated a significant difference between treatments (P = 0.005), but no significant effect for trials (P = 0.283) or the interaction between treatments of different trials (P = 0,507). There was no significant difference shown in lesion control between fruit stored at 4°C (52.6%) and -0.5°C (54.5%) (Table 3). However, cold storage in combination with the packhouse treatments showed significantly higher levels of control, with no difference between 4°C (96.3%) and -0.5°C (95.5%). The packhouse treatments without any additional cold treatment resulted in 84.2% control of latent infections. The standard deviance indicated consistent levels of control from combination treatments and more variation of the cold storage treatments alone.

Less than 0.35% of lesions, as observed in the control treatment, developed pycnidia (Table 3). The full packhouse treatment combined with -0.5°C cold storage reduced this significantly to 0.16%, whilst the other treatments had intermediate levels of reproductive lesions (0.195-0.292%).

Pycnidiospore release and spore viability

Successful pycnidiospore release from pycnidia was achieved. Spore release initiation from pycnidia present in lesions ranged from 1 min to 5 mins. Spore release initiation from pycnidia present in hard spot and cracked spot lesions only started 7 min after a droplet was placed on

a lesion, taking up to 20 minutes with older lesions. An example of pycnidia releasing pycnidiospores is shown in Figure 1.

Spores were harvested from oozing pycnidia, but due to contamination in >60% of the OA plates, results from these spore viability tests could not be analysed. Investigation into the source of the contamination indicated that it originated from the lesions themselves. After 2 weeks' incubation at high humidity and 25°C, many saprophytes and other opportunistic pathogens established in the dead tissue that forms part of the CBS lesions. Different chemicals were used to sanitize the lesion (3% sodium hypochlorite solution, 2 minute dip in 70% ethanol, chlorine wash, ozone wash), but none were successful. The method to test pycnidiospore viability therefore needs to be refined and developed further.

During oozing trials, it was observed that lesions covered with an intact wax coating did not release spores, as the wax appeared to be physically restricting spore release. This applied to the individual wax treatment and combination treatments.

Verification of lesion diagnosis

In all the PCR cycles the positive control tested positive and the negative control tested negative, showing both the extraction and PCR run was executed correctly. In summary, 93.7% of all the samples tested positive for *P. citricarpa*. A single lesion from lemons tested negative, while the remainder (n = 8) were lesions from oranges. The negative samples collected from Valencia oranges were subsequently identified as *Colletotrichum gloeosporioides*, a fungus that creates very similar lesions to that of *P. citricarpa* and which is commonly co-isolated from CBS lesions.

Residue analysis

The range of residue levels for the various fungicides that were used in the different treatments is given in Table 4. These residue results indicated that all treatment applications yielded expected residue results when following industry-simulated applications, and showed that MRL's were not exceeded for any of the fungicides used in these trials. Low levels of PYR contamination were observed in a few wax treatment samples. The source of this contamination was confirmed to be the rollers that form part of the forced air drying tunnel.

DISCUSSION

In these trials, we made use of natural infection, which could not be quantified before treatment application. The trial setup needed to account for the unknown infection levels present on the fruit so that results could be interpreted in terms of control. Since it could not be quantified accurately prior to each trial, possible variation was addressed by increasing the sample size

of fruit used in each treatment. We also tripled the sample size of the control fruit to determine a more accurate level of infection present on fruit. Whilst latent infection levels in the control treatments varied markedly between trials, and between Eureka lemons and Valencia oranges, similar trends in control of latent infections following the various treatments were observed. In the PCR analysis of the subsample of each trial, > 93% tested positive for *P. citricarpa,* showing that visual symptom diagnosis was accurate.

Incubation conditions employed presented optimal conditions for development of latent CBS infections as well as pycnidium formation, *i.e.* warm temperature and high humidity (Kiely, 1948; Baldassari *et al.*, 2007; Wang and Dewdney, unpublished results). Evaluations were conducted after 7 and 14 days' incubation, and whilst markedly higher numbers of lesions were recorded on day 14. This assessment was often problematic due to development of postharvest decay. Nevertheless, results based on the 7- and 14-day assessment were found to correlate with regard to increased lesion counts in control fruit, with similar levels of control recorded from treatments applied.

The cold storage treatment showed low and variable levels of control of latent infections on lemons (32.5%) and oranges (12.1%) in the initial trials. However, in the cold sterilization trials treatments showed moderate control of latent infections (52.6-54.5%), albeit at variable levels. Previous studies conducted by Agostini *et al*. (2006) indicated that cold storage (8°C) reduced the postharvest incidence of CBS lesions on fruit. Agostini hypothesised that the lower temperatures retard fungal growth, and in turn, symptom development.

Results from the pre-packhouse drench for both lemons and oranges showed that the treatment had no significant control of latent infections. Korf *et al.* (2001) demonstrated a high level of *in vitro* inhibition of *P. citricarpa* by TBZ and GZT, both used in the preharvest drench in this study, but also found no significant inhibition when evaluating these fungicides in *in vivo* trials. This discrepancy shows that *in vitro* studies can only be used as an indicator of a chemical's potential use against a pathogen, and are not necessarily indicative of *in vivo* efficacy.

The chlorine wash showed low and variable control of latent infections on lemons (16.8%), but showed moderate and variable levels of control when applied to oranges (46.0%). Korf *et al*. (2001) showed that chlorine had no effect on CBS infection (visible lesions) on fruit.

The efficacy of IMZ dip treatments in controlling latent infections when applied to Eureka lemons ranged between -4.6 to 53.5%, with high variability, depending on the temperature of application. However, on Valencia oranges IMZ dip treatments showed significant and more consistent control of latent infections, with no significant difference between application temperatures (69.4-81.5%). Unpublished results from research conducted by M.D. Laing indicated the effect of brief hot water dips (68°C for 20 s) gave some level control of latent

infections. Whilst the IMZ dips in this study was conducted at lower temperatures, a temperature effect was not observed.

The wax treatment showed moderate to good (46.4% on Eureka lemons and 61.7% on Valencia oranges) control of latent infections. This supports the study by Seberry *et al.* (1967) which found that by applying a wax coating to Valencia fruit symptom development on stored fruit was significantly reduced. *Phyllosticta citricarpa* is essentially an endophyte, expressing lesions only as the fruit becomes weaker (Kiely, 1948; Kotze, 1981). By delaying fruit ripening and aging using wax and cold storage, the pathogen development can be suppressed. Furthermore, an important observation was that the wax coating restricted the release pycnidiospores, which is a key consideration since exported fresh fruit is treated with a wax coating. The wax coating restricts moisture loss and physiological ageing (Deetlefs, 1959; Long and Leggo, 1959).

The combination of packhouse treatments consistently showed significant control of latent CBS infections on Valencia oranges (56.1%) and Eureka lemons (58.4%). Placing fruit into cold storage after the application of the full packhouse treatment further improved the levels of control (71.4 - 73%). This observation was confirmed when comparing the combination of treatments alone (84.2%) and with standard cold storage (4°C) with cold-sterilisation cold storage (-0.5°C) (95.6-96.3% control). Agostini *et al.* (2006) showed the controlling effects of cold storage, but found no consistent control following packhouse treatments. However, our findings confirm those of Korf *et al.* (2001) where a 3- to 7-fold reduction in lesion viability was demonstrated following the combined packhouse treatments. However, isolations from hard spot lesions in our study did not indicate any control relative to untreated fruit.

It is evident from all the trials conducted that *P. citricarpa* from expressed postharvest CBS lesions have a very low reproductive potential. Regardless of the trial and type of treatment applied to fruit, the percentages of lesions that developed pycnidia were extremely low: 0.001 – 2.09% on Eureka lemons and 0 – 0.35% on Valencia oranges. Korf *et al.* (2001) showed variable effects of the individual postharvest treatments on the viability of pycnidiospores harvested from hard spot lesions on treated fruit, viable spores could not be harvested from fruit treated in the combined treatments. Due to high levels of contamination, pycnidiospore viability testing could not be performed in our study.

Results from our study and those presented by Korf *et al.* (2001) showed variable levels of control by individual packhouse treatments, but more consistent and higher levels of control following the combined treatments, especially when combined with cold storage. This indicates cumulative or synergistic effects of these treatments against latent and visible CBS infection. The combination treatment with cold storage is the standard packhouse treatment for fresh citrus fruit, and it could therefore be accepted that CBS lesion that were present in

the packhouse, and particularly those that develop postharvest will have very poor reproductive ability, even when incubated at highly suitable conditions.

CONCLUSION

Individual postharvest fungicide treatments showed variable CBS control, but the combination treatments consistently resulted in significant control of latent infections. Lesion viability studies showed that treating fruit displaying visible lesions with postharvest fungicides did not affect lesion viability. Any affected fruits should be removed during sorting for product quality reasons. The IMZ dip resulted in variable control of latent infection. The full packhouse treatment exerted significant control on new lesion development and pycnidia formation. The wax application retards spore release by acting as a barrier and has moderate to significant control of latent infections.

The lesions that develop on harvested and packhouse treated fruit from latent infections have very poor reproductive capability. The trials showed that the percentages of lesions that developed pycnidia were extremely low. The epidemiological requirements for pycnidiospore release and movement, is the presence of water. Furthermore, pycnidiospores rely on water to move over short distances (<1m), and long wetness periods on susceptible host tissue for infection (Kiely, 1948; McOnie, 1965; Spósito et al., 2008, 2011). This combination of environmental requirements indicate that harvested fruit is not an epidemiologically significant pathway for spread of CBS, as was also concluded by previous CBS pest risk assessments (South African CBS PRA, 2000-2009; USDA-APHIS, 2010).

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TABLES AND FIGURES

Table 1: Mean percentage control (with standard deviation) of latent *Phyllosticta citricarpa* infections on Eureka lemons and percentage of newly formed lesions that produced pycnidia, as observed 6 weeks (including a 1-week incubation period) after treatment with various standard individual and combination postharvest treatments.

Treatment [a]	Percentage control of latent infections (standard deviance) [b] Day 7		Percentage control of latent infections (standard deviance) [b] Day 14		Percentage reproductive lesions [b] Day 7		Percentage reproductive lesions [b] Day 14	
Control Ambient	-		-	-	**1.06**	**ab**	**0.10**	**bcd**
Cold storage (7°C)	**32.5** (49.76)	**bcd**	**47.34** (31.27)	**bc**	**0.39**	**b**	**0.20**	**a**
Pre-packhouse drench	**9.51** (50.62)	**de**	**26.43** (47.67)	**cd**	**0.41**	**b**	**0.07**	**de**
Chlorine wash	**16.77** (68.82)	**de**	**26.68** (63.91)	**cd**	**0.04**	**b**	**0.07**	**de**
IMZ Dip at 25°C	**53.54** (49.30)	**abc**	**59.55** (38.86)	**ab**	**2.10**	**a**	**0.20**	**ab**
IMZ Dip at 35°C	**-4.56** (60.45)	**e**	**6.67** (56.53)	**d**	**0.57**	**b**	**0.17**	**abc**
IMZ Dip at 45°C	**27.85** (58.35)	**cd**	**29.97** (58.46)	**c**	**0.03**	**b**	**0.08**	**cde**
IMZ Dip at 55°C	**51.89** (39.16)	**abc**	**62.76** (26.26)	**ab**	**0.40**	**b**	**0.06**	**de**
Wax	**46.43** (43.70)	**abc**	**56.85** (28.08)	**ab**	**0.36**	**b**	**0.15**	**abcd**
Combination (Ambient)	**58.36** (30.56)	**ab**	**68.43** (28.36)	**ab**	**0.32**	**b**	**0.02**	**e**
Combination (Cold 7°C)	**71.43** (24.67)	**a**	**76.91** (12.49)	**a**	**0.00**	**b**	**0.01**	**e**

[a]Combination is the full packhouse treatment, IMZ Dip refer to the fungicide bath, control ambient was used to calculate percentage control hence no value,

[b] Mean percentages followed by the same letter in each column do not differ significantly per Fisher's least significant difference test ($P < 0.01$; LSD= 27.054 and 1.184, respectively)

Table 2: Mean percentage control (with standard deviation) of latent *Phyllosticta citricarpa* infections on Valencia oranges and percentage of newly formed lesions that produced pycnidia, as observed 6 weeks (including a 1-week incubation period) after treatment with various standard individual and combination postharvest treatments.

Treatment [a]	Percentage control of latent infections (standard deviance) [b] Day 7		Percentage control of latent infections (standard deviance) [b] Day 14		Percentage reproductive lesions [b] Day 7		Percentage reproductive lesions [b] Day 14	
Control Ambient	-	-	-	-	**0.06**	**bc**	**0.03**	**cd**
Cold storage (7°C)	**12.10** (58.70)	**d**	**8.26** (59.22)	**e**	**0.15**	**ab**	**0.13**	**bc**
Pre-packhouse drench	**2.04** (66.49)	**d**	-**6.14** (58.64)	**e**	**0.06**	**bc**	**0.01**	**cd**
Chlorine wash	**46.04** (41.57)	**c**	**31.55** (52.40)	**d**	**0.05**	**bc**	**0.34**	**a**
IMZ Dip at 25°C	**69.36** (27.42)	**ab**	**62.89** (34.39)	**bc**	**0.10**	**abc**	**0.16**	**b**
IMZ Dip at 35°C	**74.36** (17.74)	**ab**	**73.24** (21.87)	**bc**	**0.17**	**a**	**0.10**	**bcd**
IMZ Dip at 45°C	**81.54** (13.47)	**a**	**78.32** (12.62)	**ab**	**0.02**	**c**	**0.05**	**bcd**
IMZ Dip at 55°C	**76.83** (13.89)	**ab**	**67.96** (19.27)	**bc**	**0.03**	**c**	**0.06**	**bcd**
Wax	**61.66** (30.57)	**abc**	**62.18** (31.95)	**bc**	**0.01**	**c**	**0.01**	**cd**
Combination (Ambient)	**56.10** (49.15)	**bc**	**54.10** (43.38)	**c**	**0.00**	**d**	**0.02**	**d**
Combination (Cold 7°C)	**72.98** (65.60)	**ab**	**97.31** (3.86)	**a**	**0.00**	**d**	**0.01**	**d**

[a]Combination is the full packhouse treatment, IMZ dip refer to the fungicide bath, control ambient was used to calculate percentage control hence no value.

[b] Mean percentages followed by the same letter in each column do not differ significantly per Fisher's least significant difference test ($P < 0.01$; LSD= 21,008 and 0,099, respectively)

Table 3: Mean percentage control (with standard deviation) of latent *Phyllosticta citricarpa* infections on Valencia oranges and percentage of newly formed lesions that produced pycnidia, as observed 6 weeks (including a 1-week incubation period) after treatment of full packhouse treatment and 5-week cold treatment at various temperatures.

Treatment [a]	Percentage control of new lesions (standard deviance) [b]		Percentage reproductive lesions [c]	
Control Ambient	-	-	0.35	a
Combination (Ambient)[1]	84.2 (18.12)	ab	0.29	ab
Cold Storage (4°C)	52.57 (58.05)	c	0.23	ab
Combination (4°C)	96.34 (7.47)	a	0.20	ab
Cold Storage (-0,5°C)	54.45 (47.46)	bc	0.28	ab
Combination (-0,5°C)	95.55 (8.85)	a	0.16	b

[a] Combination is the full packhouse treatment, control ambient was used to calculate percentage control hence missing value.

[b] Showing the combined results of three trials conducted on Valencia oranges. Mean percentages followed by the same letter in columns do not differ significantly per Fisher's protected t-test least significant difference (P ≤0.05; LSD= 29,966).

[c] Showing the combined results of three trials conducted on Valencia oranges. There is no significant difference between treatments per Fisher's protected t-test least significant difference (P = 0,18 LSD= 0,156).

Table 4: Range of residue levels detected on Eureka lemons and Valencia oranges following various standard individual and combination postharvest treatments.

Trial	Treatments	Residue mg/kg [a]			
		Pyrimethanil [1]	**Thiabendazole [2]**	**2,4-D [3]**	**Imazalil [4]**
Eureka lemons / Valencia oranges					
	Control	0	0	0	0
	Pre-packhouse drench	0.63 - 4.03	0.19 - 2.24	0.12 - 0.67	0
	IMZ Dip 25°C	0	0	0	0.17 - 1.82
	IMZ Dip 35°C	0	0	0	0.21 - 1.77
	IMZ Dip 45°C	0	0	0	0.25 - 2,46
	IMZ Dip 55°C	0	0	0	0.69 - 4,62
	Wax	0 - 0,81	0.01 - 4,93	0,1 - 0.61	1,98 - 4,25
	Combination 1 (Ambient)	0.69 - 3,17	0.14 - 4.91	0.15 - 0,60	2.21 - 4.96
	Combination 1 (7°C/ 4°C)	0.85 - 5,0	0.13 - 5.00	0.12 - 0.77	1.27 - 4,87
Cold Storage (Valencia oranges)					
	Control	0	0	0	0
	Combination 1 (Ambient)	1.02 - 1.38	2.20 - 2.59	0.1 - 0.12	0.20 - 2.94
	Combination 1 (4°C)	1.16 - 2.11	1.89 - 3.39	0.09 - 0.14	2.47 - 3.01
	Combination 1 (-0.5°C)	1.19 - 2,14	2.03 - 2.80	0.10 - 1.0	2,59 - 2.88

[a] The maximum residue range shown here includes, the lowest MRL found out of all the trials to the highest MRL found for a specific treatment.

[1] Pyrimethanil has a maximum residue limit of 8 mg/kg, when exporting to the EU.

[2] Thiabendazole has a maximum residue limit of 5 mg/kg, when exporting to the EU.

[3] 2,4-D has a maximum residue limit of 1 mg/kg, when exporting to the EU.

[4] Imazalil has a maximum residue limit of 5 mg/kg, when exporting to the EU.

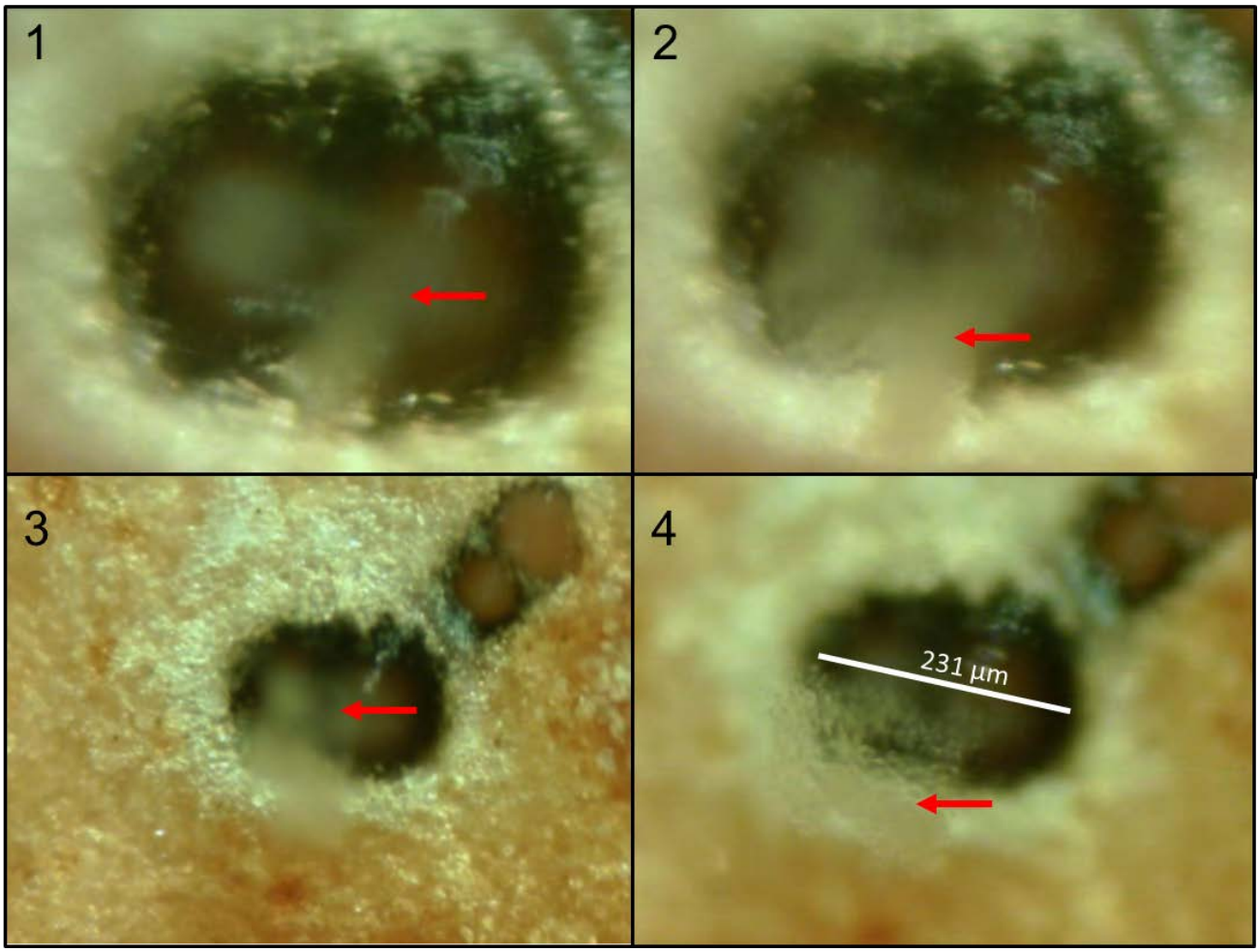

Figure 1: Photo plates depicting the progressive release of hyaline pycnidiospores from two adjacent pycnidia in two virulent spots (photos 1 and 2, and 3 and 4, respectively). The spores are released in a gelatinous mass oozing from the pycnidia, visible as an increasingly blurred area (red arrows). In photo 4 individual pycnidiospores can be discerned, with the combined diameter of the two pycnidia indicated as 231 µm.

CHAPTER 3

Effects of packhouse treatments and alternative compounds on the viability and reproductive ability of citrus black spot lesions

ABSTRACT

Phyllosticta citricarpa, causal agent of citrus black spot (CBS) is a leaf and rind-borne disease with serious phytosanitary ramifications for citrus exporters. This fungal disease is especially important when exporting to specific markets, such as the European Union where phytosanitary trade restrictions against fruit from production areas where this organism occurs have been imposed. Following the trials conducted in 2015, and after the loss of guazatine (GZT) MRL for postharvest use on all citrus exported to EU markets, research was continued to find substitutes that could be incorporated into the already established postharvest treatment regimes. Alternative compounds with MRL's accepted in the EU was selected and the full packhouse treatment, with the 2015 protocol repeated in 2016. Offered as a suitable alternative for GZT, propiconazole (PPZ) was introduced in the South African postharvest industry recommendations, and the compound was therefore used as substitute for GZT. There was no significant difference in terms of CBS control between fruit that received the 2015 or 2016 full packhouse treatment. As in 2015 trials, fruit that received the full packhouse treatment, along with a cold storage period (Valencia oranges 4°C, Eureka lemons 7°C), showed significant control of both lesions and pycnidia formation. Three alternative single treatments showed potential to control latent infections: fludioxonil (FLU), potassium sorbate and Propirly 270 EC (PPZ + Pyrimethanil (PYR)). Fludioxonil and Propirly 270 EC treatments resulted in moderate to significant control of latent infections in both Valencia oranges and Eureka lemons. Potassium sorbate moderately controlled latent CBS infections in both Valencia oranges and Eureka lemon trials. Due to climatic conditions, disease incidence for the 2016 production season was extremely low. Although some of the fungicides used in this trial can potentially control latent CBS infections, further studies over more production seasons are required before recommendations can be made.

INTRODUCTION

Citrus black spot (CBS) is not a postharvest fruit rotting disease, causing superficial lesions on fruit rind. However, under highly suitable conditions, high levels of infection can sometimes lead to premature fruit drop (Whiteside, 1993). The perceived economic threat posed by CBS had led to it being declared as an A1-quarantine organism by the European Union (Directive 98/2/EC) despite numerous reports to the contrary (Truter, 2010; CBS Expert Panel, 2013; 2014; 2015).

CBS is a monocyclic disease (Agrios, 2005). Furthermore, *Phyllosticta citricarpa* (McAlpine) van der Aa has two distinct life cycles: a teleomorph stage, which acts as the main source of inoculum in the orchard, producing ascospores from pseudothecia that develop and mature in leaf litter (McOnie, 1965; Fourie *et al.*, 2013). The second stage is anamorphic and the secondary inoculum source, producing pycnidiospores from pycnidia on certain fruit, leaf or twig lesions. Whilst the epidemiological contribution of the anamorphic or asexual stage is regarded as minor, it is more important in lemon that has overlapping fruit sets (Kiely, 1948b; Kotzé 1963, 1981; Sutton and Waterson, 1966) and in countries with highly suitable climates (Spósito *et al.*, 2007, 2011).

Most commercially grown citrus varieties are susceptible to infection with *P. citricarpa*, with Eureka lemons and Valencia oranges being the most susceptible (Kiely, 1948a, 1970; Brodick, 1969). As per packhouse regulations, fruit expressing any lesions are removed from the packline during sorting. However, the biggest threat to export is the presence of latent infections that can remain asymptomatic for a long period and only be expressed after packaging and shipping. Should any fruit expressing lesions be found in a consignment of fruit by countries that has imposed CBS phytosanitary restriction, the whole consignment may be rejected, resulting in financial losses (CGA, 2016).

In 2015 trials were conducted to evaluate the efficacy of the standard packhouse fungicidal, sanitation and cold storage treatments, as singular and combined treatments, in suppressing latent CBS infections. These studies included GZT, a highly active fungicide against sour rot, caused by *Geotrichum citri-aurantii (Ferraris) E.E. Butler*. However, early in the 2016 season the use of this compound in citrus packhouses was constrained due to the European Union lowering the maximum residue level (MRL) to 0.05 $mg.kg^{-1}$ (European Commission, 2016). Propiconazole was subsequently deployed to control postharvest sour rot infections (McKay *et al.,* 2012). As a postharvest fungicide against sour rot, PPZ is a relatively new registration in South Africa for use on citrus. The full packhouse treatment of 2015 needed to be repeated, and had to include alternative chemicals that could be incorporated into the existing packhouse regime, as well as

other actives that had suitable MRL on citrus to allow postharvest use. To this end, PPZ, as the replacement for GZT, was added to the list of new alternatives to be tested.

Propiconazole was first registered in 1988 for use on seed grass and later on cereals for control of preharvest diseases (Worthing, 1983; Thomson, 1997; European Protection Agency, 2006), and has mainly been used as a preharvest application. It is a triazole regarded as a medium risk fungicide group (FRAC, 2016), and functional as a systemic fungicide that has both protective and curative activity (Worthing 1983; Thomson, 1997). No previous studies have been conducted to determine whether PPZ is effective against CBS infection.

Fludioxonil, another chemical that has not previously been widely used in the South African citrus industry, is a synthetic analogue of the bacterial metabolite pyrrolnitrin. It was first evaluated for postharvest use as an alternative to IMZ (Schirra *et al.,* 2005; Zhang and Timmer, 2007; Förster *et al*., 2007). The active ingredient causes inhibition of mycelium growth, lower osmotic signal transduction leading to inhibition of spore germination and germ tube elongation (Rosslenbroich and Stuebler, 2000). As a broad spectrum, non-systemic fungicide with long residual activity (PMEP, 2016), and, because it has been considered as an alternative to IMZ it was prudent to include this fungicide in the trials. Fludioxonil has been used in CBS trials conducted by Agostini *et al*. (2006), but these studies showed that no single fungicide application had any measurable effect on inhibiting lesion formation.

Sodium carbonate (Na_2CO_3) is a GRAS (generally regarded as safe) chemical acceptable to use in all food products (Lakhanisky, 2012) and has been used as an antimicrobial agent. The compound can be used on all fruit exported to the EU, with no MRL limits (European Commission, 2016). Prior to the trials conducted in this study there was no available literature indicating that sodium carbonate has previously been used to control infection by the CBS fungus.

Another GRAS chemical used in citrus packhouses as a postharvest decay remedy is potassium sorbate. Unfortunately, it's not allowed to be used on any food exported to the EU (European Commission, 2016), but is known to inhibit the growth of a broad spectrum of microorganisms (Mendonca, 1992). No previous studies have been conducted to evaluate the effect of potassium sorbate on latent citrus black spot infections.

A new product recently introduced for postharvest use is Fortisol CA Plus (Productos Citrosol S.A., Partida Alameda Parc. C. 46721 Potries (Valencia), Spain), which contains water-soluble salts calcium, potassium and phosphorus. The developers of this product claim that it stimulates the fruit's natural defences against active pathogens. This was the incentive for including Fortisol in the trials against latent CBS infection. Other than the marketing material, there was limited literature available regarding the claims and purported efficacy of this product (Citrosol, 2016).

The objective of this study was to evaluate alternative fungicides and GRAS chemicals that can possibly be incorporated into a control programme against latent CBS infection.

MATERIALS AND METHODS

Fruit used in trials

Eureka lemons and Valencia oranges were used in all the trials. These cultivars were chosen for their high susceptibility to infection by *P. citricarpa* (Kiely, 1948a). Fruit used in the trials were harvested from farms located in Gauteng and Mpumalanga provinces. Lesion-free fruit were collected from unsprayed or abandoned orchards that were verified to have CBS infection following prior inspection. Fruit were harvested into plastic fruit crates (325 x 505 x 245 mm) and transported to the laboratory. Fruit from seven different orchards were used: three Eureka lemon and four Valencia orange orchards.

Postharvest treatments

The full packhouse treatment consisted of the pre-packhouse drench (mixture 1 or 2), chlorine wash, IMZ dip at 35°C, and a wax application, and evaluated as the combination 1 (with GZT) and combination 2 (with PPZ instead of GZT).

Pre-packhouse drench (mixture 1 and 2)

In South Africa fruit are drenched before degreening to minimize postharvest decay, primarily caused by *Penicillium* spp. and *Galactomyces* spp. (Ladaniya, 2010). Two drench mixtures were prepared, one containing GZT and the other containing PPZ. The drench mixture 1 contained TBZ (1000 $mg.L^{-1}$), PYR (1000 $mg.L^{-1}$), GZT (1000 $mg.L^{-1}$) and 2,4-D (250 $mg.L^{-1}$) was prepared by filling a container with 50 L of tap water and adding fungicides in the order that follows, while constantly stirring the mixture to keep all the fungicides in suspension: 100 mL TBZ (Thiabendazole, 500 $g.L^{-1}$ SC, ICA International Chemicals, Stellenbosch, South Africa), 125 mL PYR (Protector, 400 $g.L^{-1}$ SC, ICA International Chemicals, Stellenbosch, South Africa), 240 mL GZT (CitriCure, 210 $g.L^{-1}$ SL, ICA International Chemicals, Stellenbosch, South Africa) and 500mL 2,4-D (2,4-dichlorophenoxy acetic acid) (Deccomone, 25 $g.L^{-1}$ SL, Citrashine, Johannesburg, South Africa).

Drench mixture 2 contained the same compounds, but PPZ (600 $mg.L^{-1}$) was used as a replacement for GZT: 250 mL of a formulated product containing both PYR and PPZ (Propirly 270 EC, PYR: 150 $g.L^{-1}$, PPC 120 $g.L^{-1}$, ICA International Chemicals, Stellenbosch, South Africa) was used, and resulted in a 750 $mg.L^{-1}$ PYR and 600 $mg.L^{-1}$ PPC solution.

A simulated dip-drench system was used (Christie, 2016). Fruit were packed into plastic crates (dimensions: 325 x 505 x 245 mm) which were used to simulate the standard 800 L orchard bin used by commercial citrus packhouses. The drench solution was applied at a rate of 12.5 L/min per crate (Christie, 2016) at an exposure time of 1 min (a dosage similar to commercial drench applicators), and then left to dry for 24 hours before other treatments were applied, before incubation.

Chlorine wash/dip

The wash solution was prepared by adding 15 g of calcium hypochlorite (HTH Pool Chlorine, 680 $mg.kg^{-1}$, WP) to 50 L of tap water to give a 200 $mg.L^{-1}$ solution. Hydrochloric acid was used to adjust the pH to 6.5 -7.5 and confirmed by pH meter (Jenway Model 3310; Bibby Scientific Limited, Staffordshire, UK). To simulate the wet dump sanitation system, fruit were submerged in this solution for 90 seconds and then left to dry.

Fungicide bath

An IMZ solution was prepared by dissolving 80.4 g of imazalil sulphate (Imazacure, 750 $g.kg^{-1}$ SG, ICA International Chemicals, Stellenbosch, South Africa) in 120 L of tap water to give a 500 mg/L solution. The pH was constantly measured with a pH meter (Jenway Model 3310; Bibby Scientific Limited, Staffordshire, UK) and kept between 3.5 – 4 by adding hydrochloric acid. This was done to keep residue loading constant at different application temperatures (Erasmus *et al.,* 2013). Citrus fruit were submerged in the solution for 60 seconds and then put over revolving brushes, as is the common practice in packhouses. The equipment used for the dip application was an experimental packline developed and custom built to simulate commercial packlines.

Wax application

A smaller version of a commercial waxing unit (Decco Citrashine (Pty) Ltd, South Africa, Johannesburg) was used for application of a polyethylene-based wax (PolyOrange, 18% solids, Decco Citrashine (Pty) Ltd, Johannesburg, South Africa). The wax suspension consisting of IMZ (2000 mg. L^{-1}), TBZ (4000 mg. L^{-1}) and 2,4-D (250 mg. L^{-1}) was prepared by pouring 25 L of wax into the mixing tank and adding the treatment chemicals to the wax while the pump was constantly mixing the solution. The individual fungicides were first dissolved in 100 mL of warm water (40°C), before adding them to the mixing tank in the following order: 100 mL of IMZ (Imazacure, 500 $g.L^{-1}$ EC, ICA International Chemicals, Stellenbosch, South Africa), 200 mL of TBZ (Thiabendazole, 500 $g.L^{-1}$ SC, ICA International Chemicals, Stellenbosch, South Africa) and 250 mL 2,4-D (2,4-

dichlorophenoxy acetic acid) (Deccomone, 25 g.L^{-1} SL, Decco Citrashine (Pty) Ltd, Johannesburg, South Africa).

The wax applicator tank was filled and left to agitate for 15 minutes before treatments commenced. The wax brushes were optimally wetted (damp to the touch) before use, and the wax applicator calibrated to administer 1 - 1.2 L of wax per ton of fruit.

Cold storage

Valencia oranges and Eureka lemons were placed into cold storage as a single treatment, and as part of the combination treatment. Eureka lemons are more prone to cold damage at low temperatures, therefore lemons were stored at 7°C and Valencias, less prone to cold damage, were stored at 4°C for 5 weeks.

Alternative fungicides

All alternatives fungicides were applied at recommended dosages as per their registered labels or commercial recommendations. Fruit were submerged in 5-L solutions containing:

- 12 mL of propiconazole (Propicure 250 EC, ICA International Chemicals, Stellenbosch, South Africa) (600 mg. L^{-1}),
- 13 mL of fludioxonil (Tutor 500 SC, ICA International Chemicals, Stellenbosch, South Africa) (600 mg. L^{-1}),
- 12 mL PPZ + PYR (Propirly 270 EC, ICA International Chemicals, Stellenbosch, South Africa) (750 mg. L^{-1} pyrimethanil, 600 mg. L^{-1} propiconazole),
- 100 mL water-soluble salts of Ca, K and P (Fortisol CA Plus, Citrosol, Partida Alamed, Spain) (2% solution, as per Citrosol recommendation),
- 50 g potassium sorbate (1% solution, Smilanick *et al.,* 2008), or
- 100 g sodium bicarbonate (2% solution, Palou *et al.*, 2016).

The solutions were prepared by adding the individual chemicals to a bucket filled with 5 L of tap water at 35°C. Fruit were submerged for 90 seconds before placing them in plastic fruit crates (325 x 505 x 245 mm) and allowing them to dry off. Since most PPZ is removed through brushing, no fruit was subjected to drying by brushing, in order to keep all the treatment implementation, the same.

Residue analysis

Six fruit were sampled from replication one and replication four, after each treatment application. The sampled fruit were placed in residue bags and deep frozen (-20°C), then later used to prepare

pulp for residue analysis. The fruit inside residue bags were defrosted, measured and weighed. The fruit were then chopped and macerated to a fine pulp using a blender (Salton Elite, Amalgamated Appliance Holdings Limited, Reuven, South Africa) and the pulp was then re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (detected as chloramizole), PYR (detected as pyrimethanil) PPZ (detected as propiconazole) GZT (detected as guazatine acetate), FLU (detected as fludioxonil), TBZ (detected as thiabendazole) and 2,4-D (detected as the free acid) residue analyses by Hearshaw and Kinnes Analytical Laboratory (Cape Town, South Africa). The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, CA, USA). The data collected and the range at which fungicide residues were loaded were determined to confirm that no maximum residue limits (MRL) were exceeded (SAS Institute Inc. Cary, NC, USA).

Trials and evaluations

In this study 11 treatments were used, with each treatment repeated four times, and each replicate consisting of 12 fruit. For residue analysis, extra fruit was included in the first and fourth replicates of each treatment.

Effects of combination and alternative treatments on latent infections

The trials were repeated three times on Eureka lemons and four times on Valencia oranges. The alternative compounds were applied as single treatments of PPZ, Propirly (PPZ and PYR), FLU, Fortisol CA PLUS (water-soluble salts of Ca, K and P), potassium sorbate, sodium bicarbonate and wax-only (polyethylene wax) and evaluated.

The full packhouse treatment consisted of the pre-packhouse drench (mixture one or two), chlorine wash, IMZ dip at 35°C, and a wax application, and evaluated as combination 1 (with GZT) or combination 2 (with PPZ) treatment. In this case, the pre-packhouse drench application was applied 24 hours before any of the other treatments to simulate the practices followed in a commercial packhouse. Both combination treatments were done in duplicate, with and without cold storage.

After treatments were applied, fruit were packed into lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa). Each carton was covered with a transparent polyethylene bag and sealed with a cable tie to ensure that a near-100% RH was maintained.

The single treatments (alternatives) and one of each of the combination treatments were stored at room temperature (22°C, 79-85% RH) for 5 weeks, along with an untreated control. A duplicate of the full packhouse treatment was placed into cold storage for 5 weeks, also along with individual cold storage treatment.

Evaluation

Lesion expression and reproductive ability

After 5 weeks, fruit stored at cold or ambient conditions were moved to an incubation room. The incubation room was optimised to enhance lesion expression, with the temperature of 25-27°C, 24-hour lighting and RH > 85%. The fruit were incubated for 2 weeks while being evaluated.

The CBS lesions on each fruit were counted on day 0 (day when removed from cold or ambient storage) and days 7 after having been placed in the incubation room. The number of lesions that developed pycnidia was also recorded.

Verification of lesion diagnosis

As per the methods described by Hu *et al.* (2013), real-time polymerase chain reaction (qPCR) was used to verify the morphological identification *P. citricarpa* from the subsamples for each trial.

DNA was extracted from the citrus black spot lesions using the PROMEGA Wizard ® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The manufacturer's instructions were slightly modified as lesion size was typically small. Pellets were re-suspended in 25 µl DNA rehydration solution. Control samples were included in the DNA extraction, including a positive fruit control (lesions from fruit that previously tested positive for *P. citricarpa*) and negative fruit control (fruit with no lesions obtained from a disease-free orchard). The elution volume was reduced to 1 × 75µL (Meyer, 2006). The extractions were placed in a 1.0% agarose gel with TBE buffer.

Species-specific real-time primers were used to amplify the ITS-region of *P. citricarpa*. The primer pair, GCITSF (5'- CCT GAA AGG TGA TGG AAG GG - 3') and GCITSR (5'- CGC CAA AGC AAC ATG GTA GAT A- 3') (Hu *et al*. 2013) was used in a 20 µl reaction to amplify the region. The qPCR reaction consisted of 2x KAPA Probe Fast qPCR master mix (KAPA Biosystems, Wilmington, MA, USA), 400 nm forward and reverse primer, 200 nm probe and 6 µl of PCR grade water. The thermal cycle consisted of an initial activation step of 95°C for 3 min, then 40 cycles of 95°C for 15 s and 59°C for 30 s. Control samples were a previously positive PCR sample as positive control as well as a no-template control.

Statistical analysis

The counts of the newly formed lesions were used to calculate the percentage control of individual replicates for each treatment relative to the mean lesion expression in the untreated control within each trial. The counts of lesions containing pycnidia was expressed as mean percentage pycnidia-forming lesions for each replicate in a treatment, within each trial. The data from all the trials conducted on Eureka lemons and all the trials conducted on Valencia oranges were thereafter grouped and used in appropriate analyses of variance (ANOVA). Fisher's LSD was calculated to identify significant differences between treatments, using a confidence interval of 95%. All statistical analyses were done using statistical analysis software Addinsoft XLSTAT Version 2015.8.18 (www.xlstat.com).

RESULTS

Effects of combination and alternative treatments on latent infections present on Eureka lemons

The incubation conditions were ideal for CBS symptom and pycnidium development, but also promoted the development of postharvest fruit rotting fungi. Due to postharvest rot, one trial had to be discarded, and the 14-day rating data could not be used. The data collected on day 7 was therefore used for statistical analysis. Mean number of lesions in the control treatments ranged from 4.65 to 5.48.

ANOVA of percentage control data indicated a significant trial x treatment interaction (P = 0.041), significant effects for treatments (P = 0.001), but no significant difference between trials (P = 0.917). The interaction was attributed to variable results of some individual treatments (PPZ, sodium bicarbonate, wax-only and Fortisol CA PLUS). Means for the two trials were considered and results are presented in Table 1. The treatment efficacy can be summarised as follows: no significant control with levels ranging from 0 to 25% (wax-only), low levels of control (26 to 50%; PPZ, sodium bicarbonate, Fortisol CA PLUS); moderate levels of control (51 to 75%; cold storage, FLU, potassium sorbate, Combination 1 (ambient), Combination 2 (ambient)); and significant levels of control (76 to 100%; Propirly, Combination 1 (7°C), Combination 2 (7°C)).

Standard deviation values (Table 1) indicate that control was mostly consistent, but variable for PPZ, sodium bicarbonate, wax-only and Fortisol CA PLUS. There was no significant difference between Combination 1 (with GZT) and Combination 2 (with PPZ), indicating that the substitution does not affect the efficacy of the full packhouse treatment. The combination treatments (1 and 2), along with a cold storage (7°C) consistently resulted in significant levels of

CBS control (80%). From the single treatments, Propirly (PPZ + PYR) had the highest efficacy in control of latent CBS infections (78.8%).

ANOVA of percentage data of newly formed lesions that developed pycnidia on Eureka lemons indicated significant effects for trials ($P = 0.001$), treatments ($P < 0.0001$) and a significant interaction between trials and treatments ($P = 0.004$). The interaction was attributed to differences in disease incidence and variable results between the two trials, but the data were combined in the analysis. Less than 0.25% of newly formed lesions developed pycnidia (Table 1). The combination 1 (ambient) (0.21%), wax (0.18%), potassium sorbate (0.18%), sodium bicarbonate (0.25%) and Fortisol CA PLUS (0.22%) formed more reproductive lesions than the control (0.17%), but did not differ significantly. The most of the individual (0.05 – 0.14%) treatments had markedly lower percentages of reproductive lesions than the control treatment (0.17%), whilst the combination treatments with cold storage (0.002%) had significantly fewer reproductive lesions.

Effects of combination and alternative treatments on latent infections present on Valencia oranges

As was experienced with the lemon trials, one trial had to be discarded due to postharvest decay, and data collected on day 7 was used for statistical analysis. Due to generally unsuitable climatic conditions during period when is fruit susceptible to the CBS organism, the latent infection levels on Valencia fruit were very low. Mean numbers of lesions in the control treatments ranged from 0.025 to 1.13.

ANOVA of percentage control data indicated significant effects for treatments and trials ($P < 0.0001$), but no significant interaction between trials and treatments ($P = 0.088$). The significant difference between trials may be ascribed to differences in disease pressure with mean control observed in Trial 2 (87.2%), significantly higher than in Trials 1 and 3 (62.6-69.3%). Table 2 shows the mean percentage control observed following various treatments in the three trials conducted on Valencia oranges. The treatment efficacy can be summarised as follows: low level control with levels ranging from 26 to 50% (Fortisol CA PLUS); moderate levels of control (51 to 75%; Cold storage, sodium bicarbonate, potassium sorbate, Wax-only, Combination 1 (Ambient)) and significant levels of control (76 to 100%; Combination 2 (Ambient), FLU, PPZ, Propirly, Combination 1 (4°C), Combination 2 (4°C)).

Standard deviation values (Table 2) indicate that control was mostly consistent, but variable for Fortisol CA PLUS. There was no significant difference between Combination 1 (with GZT) and Combination 2 (with PPZ), indicating that the substitution does not affect the efficacy of the full packhouse treatment. The combination treatments (1 and 2), along with a cold storage (4°C)

consistently resulted in significant levels of CBS control (84.3 to 92.9%). From the single treatments, Propirly (PPZ + PYR) had the highest efficacy in control of latent CBS infections (100%).

ANOVA of percentage data of newly formed lesions that developed pycnidia on Eureka lemons indicated no significant effects for trials ($P = 0.44$), treatments ($P = 0.07$) or the interaction between trials and treatments ($P = 0.87$). Due to the low levels of pycnidia formation there was no significant difference between treatments. Less than 0.11% of newly formed lesions developed pycnidia (Table 2). The combination 1 (ambient) (0.03%), wax (0.1%) and Fortisol CA PLUS (0.04%) formed more reproductive lesions than the control (0.01%), but did not differ significantly. Most of the individual treatments had markedly lower percentages of reproductive lesions (0 – 0.008%) than the control treatment (0.01%), whilst the combination treatments with cold storage (0%) developed no reproductive lesions.

Verification of lesion diagnosis

PCR verification of lesion diagnosis was conducted on 77 samples. In all the PCR cycles both the positive control and negative control tested true, showing both the extraction and PCR run was executed correctly. In summary, 98.7% of all the samples tested positive for *Phyllosticta citricarpa*. One lesion from lemons tested negative, but was not identified further.

Residue analysis

The range of residue levels for the various fungicides that were used in the different treatments is given in Table 3. These residue results indicated that all treatment applications yielded expected residue results when following industry-simulated applications, and showed that MRL's were not exceeded for any of the fungicides used in these trials. This includes the GZT MRL, but it is now inconsequential due to the loss of the MRL.

DISCUSSION

Previous studies by Korf *et al*. (2001) looked at conventional chemistry used at the time, showing the usefulness of such postharvest applications. New compounds (active ingredients), GRAS chemicals and alternative combinations implemented more recently, were included in this study, and were similarly proven to be valuable in the control of CBS.

Due to the extremely low likelihood of successfully artificially inoculating citrus fruit with *P. citricarpa*, only natural infection was used in these trials. This implies that latent infections could therefore not be quantified before treatment application. The trial protocol was therefore designed

to account for the unknown infection levels present on the fruit by increasing the sample size used in each treatment, enabling results to be interpreted in terms of untreated controls. Whilst latent infection levels in the control treatments varied markedly between Eureka lemons and Valencia oranges, similar trends in control of latent infections following the various treatments were observed. In the PCR analysis of the subsample of each trial, > 98% tested positive for *P. citricarpa,* confirming that visual symptom identification was accurate.

The cold storage treatment of Valencia (58.4%) and Eureka (52%) fruit resulted in similar percentage moderate control of latent infections. Agostini *et al* (2006) postulated that the lower temperatures create an unsuitable environment, slowing fungal growth and reducing latent infections.

Single treatments with FLU (70.2 to 82.2%), Propirly (78.8 to 100%) and potassium sorbate (68.8 to 70.1%) showed consistent levels of control on both Eureka lemons and Valencia oranges. No one of the other GRAS chemicals resulted in any significant control of lesion expression. This concurs with studies by Palou *et al*. (2016) which indicated that alternative GRAS chemicals, though effective, still only offer limited postharvest disease control efficacy, and that combination of such chemicals with conventional chemistry is still necessary in the foreseeable future. The wax-only treatment showed variable results on Valencias (60%) and Eureka (21.4%), with inconsistent levels of control, as shown by standard deviation. Seberry *et al.,* (1967) showed that treating fruit with wax coating gave significant control of latent infections, which is shown by results from trials conducted on Valencia oranges.

Propiconazole gave better and more consistent control of latent infections on Valencia fruit (91.7%) than on Eureka lemons (30.5%). Treatment using Propirly 270 EC (a PYR blend with PPZ at a lower concentration) consistently resulted in significant levels of control that would suggest that the added PYR has a synergistic effect, offering improved control of latent infections. The sodium bicarbonate treatment resulted in low and variable control of latent infections on Eureka lemons (35.6%), and moderate and more consistent control on Valencia oranges (65.9%). The inconsistent treatment efficacy between cultivars can be attributed to differences in infection levels between trials and cultivars. Agostini *et al*., (2006) reported that no single treatment had any significant effect on control of latent infections.

There was no significant difference between combination 1 (with GZT) and combination 2 (with PPZ). This lead to the conclusion that the substitution of GZT with PPZ did not have an effect on the efficacy of the full packhouse treatment. Comparable with previous results from 2015 trials, the combination (1 + 2) with a cold treatment (Valencia 84.3 to 92.7%, Eureka 80%)

showed higher levels of control than treated fruit stored at ambient (Valencia 63.3 to 81.4%; Eureka 66.3 to 72.4%).

Control fruit had an overall low level of reproductive lesions (lemons 0.17%; oranges 0.01%). The combination treatments (1 and 2) along with a cold storage treatment lowered the formation of reproductive lesions by 0 – 0.002%. This study confirms that fruit lesions have a very low reproductive potential, and by applying a postharvest packhouse treatment the low reproductive potential is lowered even further.

New advances and improvements in postharvest fruit sanitation may offer additional control of latent infections and pycnidial development, and should be included in future research (Feliziani et al., 2016).

CONCLUSION

Some individual treatments showed consistent results (FLU, Propirly, potassium sorbate), others showed variable results (commercial coating wax, PPZ, sodium bicarbonate). Single treatments that gave consistent control on both fruit types have the potential to be used for the control of latent CBS infections. Due to adverse climatic conditions that lead to relatively low infection levels of the fruit, repeating the trials under conditions with high disease pressure will enhance this overview of treatment performance. The formulation that contains both PYR and PPZ (Propirly) seem to have a synergistic effect, improving control of latent infections. There is no significant difference in control efficacy between combinations where GZT was substituted with PPZ. Combination treatments (1 and 2) showed consistent and moderate to significant control of latent infections. The full packhouse treatment resulted in significant control of pycnidia formation on newly formed lesions. The results from combination treatments in 2015 trials directly correlates with results from 2016 trials.

Similarly, latent infections that developed into lesions on fruit that was treated in a packhouse have very poor reproductive capability. Results from this study showed that the percentages of lesions that developed pycnidia were extremely low, and were comparable to results from 2015 trials.

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TABLES AND FIGURES

Table 1: Mean percentage control (with standard deviation) of latent *Phyllosticta citricarpa* infections on Eureka lemons and percentage of newly formed lesions that produced pycnidia, as observed 6 weeks (including a 1-week incubation period) after treatment with various alternative individual and standard combination postharvest treatments.

Treatments [a]	Percentage control of latent infections (Standard deviance) [b]		Percentage reproductive lesions [c]	
Control Ambient	-	-	0.17	abc
Cold storage (7°C)	51.98 (21.66)	abc	0.14	bcd
Fludioxonil	70.20 (9.86)	ab	0.10	cde
Propiconazole	30.49 (61.15)	c	0.09	cde
Propirly	78.83 (11.26)	a	0.05	de
Potassium sorbate	70.08 (30.94)	ab	0.18	abc
Sodium bicarbonate	35.55 (58.82)	bc	0.25	a
Fortisol CA plus	31.15 (47.79)	c	0.22	ab
Wax-only	21.38 (42.79)	c	0.18	abc
Combination 1 (Ambient)	72.39 (19.29)	a	0.21	ab
Combination 1 (7°C)	80.00 (19.24)	a	0.002	e
Combination 2 (Ambient)	66.27 (21.01)	ab	0.09	cde
Combination 2 (7°C)	80.00 (15.87)	a	0.002	e

[a] Combination 1 (with GZT) and combination 2 (with propiconazole) is the full packhouse treatment, control ambient was used to calculate percentage control hence no value.

[b, c] Mean percentages followed by the same letter in each column do not differ significantly per Fisher's least significant difference test ($P \leq 0.05$; LSD = 34.811 and 0.109, respectively).

Table 2: Mean percentage control (with standard deviation) of latent *Phyllosticta citricarpa* infections on Valencia oranges and percentage of newly formed lesions that produced pycnidia, as observed 6 weeks (including a 1-week incubation period) after treatment with various alternative individual and standard combination postharvest treatments.

Treatments [a]	Percentage control of latent infections (Standard deviance)[b]		Percentage reproductive lesions [c]	
Control Ambient	-	-	0,010	b
Cold storage (4°C)	58.36 (30)	d	0,008	b
Fludioxonil	82.15 (17.33)	abc	0,00	b
Propiconazole	91.69 (11.29)	a	0,00	b
Propirly	100.00 (0)	a	0,00	b
Potassium Sorbate	61.84 (33.85)	cd	0,00	b
Sodium Bicarbonate	65.88 (34.67)	bcd	0,007	b
Fortisol CA plus	34.63 (52.17)	e	0,04	b
Wax-only	59.96 (22.71)	d	0,10	a
Combination 1 (Ambient)	63.30 (36.40)	cd	0,03	b
Combination 1 (4°C)	84.34 (15.18)	ab	0,00	b
Combination 2 (Ambient)	81.40 (17.78)	abc	0,00	b
Combination 2 (4°C)	92.87 (9.78)	a	0,00	b

[a] Combination 1 (with GZT) and combination 2 (with propiconazole) is the full packhouse treatment, control ambient was used to calculate percentage control hence no value.

[b, c] Mean percentages followed by the same letter in each column do not differ significantly per Fisher's least significant difference test (P ≤0.05; LSD= 20.98 and LSD= 0,109 respectively).

Table 3: Range of residue levels detected on Eureka lemons and Valencia oranges following various alternative individual and standard combination postharvest treatments.

Treatments[a]	Residue mg/kg [b]						
	Pyrimethanil[1]	Propiconazole[2]	Fludioxonil[3]	Guazatine[4]	Thiabendazole[5]	2,4 D[6]	Imazalil[7]
Control	0	0	0	0	0	0	0
Propiconazole	0	1.98 - 6.05	0	0	0	0	0
Fludioxonil	0	0	0.31 - 2.76	0	0	0	0
Propirly	2.12 - 5.67	1.11 - 3.69	0	0	0	0	0
Potassium sorbate	0	0	0	0	0	0	0
Sodium bicarbonate	0	0	0	0	0	0	0
Fortisol CA plus	0	0	0	0	0	0	0
Wax-only	0	0	0	0	0	0	0
Combination 1	0.49 - 1.6	0	0	0.23 - 3.89	1.03 - 4.13	0.11 - 0.32	0.84 - 4.36
Combination 2	0.68 - 2.05	0.38 - 1.16	0	0	0.35 - 3.90	0.04 - 0.54	1.95 - 5.0

[a] Combination 1 (with guazatine) and combination 2 (with propiconazole) is the full packhouse treatment, control ambient was used to calculate percentage control hence no value.

[b] The maximum residue range shown here includes, the lowest MRL found out of all the trials to the highest MRL found for a specific treatment.

[1] Pyrimethanil has a maximum residue level of 8 mg/kg, when exporting to the EU.

[2] Propiconazole has a maximum residue level of 9 mg/kg, when exporting to the EU.

[3] Fludioxonil has a maximum residue level of 10 mg/kg, when exporting to the EU.

[4] Guazatine no longer has a MRL in the EU (0.05)

[5] Thiabendazole has a maximum residue level of 5 mg/kg, when exporting to the EU.

[6] 2,4-D has a maximum residue level of 1 mg/kg, when exporting to the EU.

[7] Imazalil has a maximum residue level of 5 mg/kg, when exporting to the EU.